

## DESCRIPTION

METHOD FOR DIAGNOSING OR PREDICTING SUSCEPTIBILITY  
TO OPTIC NEUROPATHY

5

## TECHNICAL FIELD

The present invention relates to a set of genetic polymorphisms linked to optic neuropathy.

## BACKGROUND ART

10        Glaucoma is a major cause of blindness worldwide, and estimated approximately 67 million people suffered from some form of glaucoma. The majority of cases occur as late adult onset (typically over age 40 years) of primary open-angle glaucoma (POAG), which is the most common form of  
15        glaucoma and affects approximately 2% in white population and 7% of black population over 40 years old. POAG results in a characteristic visual field changes corresponding to the excavation of the optic disc that is usually associated with an elevation of intraocular pressure (IOP). Normal-  
20        tension glaucoma (NTG) is a form of open-angle glaucoma in which typical glaucomatous cupping of the optic nerve head and visual field loss are present but in which there is no evidence of increased IOP over 21 mm Hg at all times. In Japan, prevalence of glaucoma is approximately 3.5 % over

40 years old: POAG 0.58 % and NTG 2.04 %. Prevalence of NTG in Japanese population is high compared with that in other populations. Glaucoma is a multifactorial disorder characterized by a progressive optic neuropathy associated with a specific visual field loss, and results from the interaction of multiple genes and environmental influences, although intraocular pressure (IOP) is a major risk factor for glaucoma.

Risk factors to develop glaucoma include high IOP, age, race, positive family history, myopia, the presence of diabetes or hypertension, and genetic factors. Although the exact pathogenesis of glaucomatous optic neuropathy is remains unclear, it is generally accepted that an increased IOP is a major risk factor. Current treatment for glaucoma consists of interventions which lower IOP. However, in some patients with glaucoma, NTG or advanced stage of POAG, reduction of IOP does not prevent the progression of the disease, indicating that factors other than an increased IOP may be involved in the development or progress of glaucoma.

POAG and NTG are a heterogeneous group of conditions probably with different multi-factorial etiologies resulting in the observed patterns of neuronal loss in the optic disk. The association between glaucoma and the

presence of many systemic vascular diseases including low systemic blood pressure, nocturnal dips in blood pressure, hypertension, migraine, vasospasm, and diabetes has been reported. The presence of optic disc hemorrhages in NTG  
5 patients suggests that vascular insufficiencies are deeply involved in the development and progression of NTG. A high percentage of patients with POAG receive a wide variety of medications for coexisting disorder. Especially, systemic hypertension was the most common disorder, occurring in 48%  
10 of the total population.

Glaucoma-like morphological changes have been reported in patients with Leber's hereditary optic neuropathy (LHON) at the atrophic stage and dominant optic atrophy (DAO). Recently, the inventor has reported optic  
15 disc excavation by a quantitative analysis using Heidelberg retinal tomography (HRT) in the atrophic stage of Japanese 15 patients with LHON harboring the 11778 mutation (Mashima Y et. al., Arch Clin Exp Ophthalmol 2003; 241:75-80, the contents of the cited reference are herein incorporated by  
20 reference). LHON is a maternally-transmitted eye disease that mainly affects young adult men. Approximately 70% of patients were male. This disease usually causes severe and permanent loss of vision resulting in a visual acuity of less than 0.1. Visual field defects are present as central

or cecocentral scotomas. So far more than 20 point mutations of mitochondrial DNA (mtDNA) have been reported in LHON patients worldwide (Brown MD et. al., Clin Neurosci 1994; 2:138-145, the contents of the cited reference are  
5 herein incorporated by reference), and more than 80% of LHON patients carry one of three mtDNA mutations at nucleotide position 3460, 11778, or 14484 (Mackey DA et. al., Am J Hum Genet 1996; 59:481-485, the contents of the cited references are herein incorporated by reference).  
10 Although NTG patients were tested for the three LHON mutations of mtDNA nucleotide positions 3460, 11778 and 14484, no mutations and no defects in respiratory chain activity in skeletal muscle samples were detected (Brierley EJ et. al., Arch Ophthalmol 114:142-146 and Opial D et. al.,  
15 Graefes Arch Clin Exp Ophthalmol 239:437-440, the contents of the cited references are herein incorporated by reference).

The major difference among LHON patients with one of these mtDNA mutations is in the clinical course. The 3460  
20 and 14484 mutations are associated with better visual prognosis than the 11778 mutation which shows visual recovery rates of only 4% to 7% (OostraRJ et. al., J med Genet 1994;31:280-286, Riordan-Eva P et. al., Brain 1995; 118:319-337, Mashima Y et. al., Curr Eye Res 1998;17:403-

408, the contents of the cited reference are herein incorporated by reference). However, visual recovery has been documented in some patients with the 11778 mutation and an age of onset in the low teens (Stone EM et. al., J clin Neuro-Ophthalmol 1992; 12:10-14, Zhu D et. al., Am J Med Genet 1992; 42:173-179, Salmaggi A et. al., Intern J Neuroscience 1994; 77:261-266, Oostra RJ et. al., Clin Genet 1997; 51:388-393, Mashima Y et. al., Jpn J Ophthalmol 2002; 46:660-667, the contents of the cited references are herein incorporated by reference). Recovery of vision appears to be more likely when visual deterioration begins at an early age, even in patients with the 11778 mutation.

The clinical variability of LHON patients, which includes age at onset, male predilection, incomplete penetrance, and visual recovery, suggests that the disease most likely results from polygenic or multifactorial mechanisms, possibly involving environmental stressors, X-chromosomal loci, and other mtDNA mutations (Man PYW et. al., J Med Genet 2002; 39:162-169, the contents of the cited reference are herein incorporated by reference). However, attempts to identify a relevant locus on the X-chromosome have not been successful (Chalmers RM et. al., Am J Hum Genet 1996;59:103-108 and Pegoraro E et. al., Am J Med Genet 2003;119A:37-40, the contents of the cited

reference are herein incorporated by reference). So-called "secondary LHON mutations" are more frequently found in European LHON patients than in unaffected Europeans and are polymorphisms linked to the European haplotype J. These polymorphisms are not strong autonomous risk factors (Brown MD et. al., Am J Hum Genet 1997;60:381-387 and Torroni A et. al., Am J Hum Genet 1997;60:1107-1121, the contents of the cited reference are herein incorporated by reference).

Thus, the primary mutations are the major risk factors in LHON, but additional etiologic factors that augment or modulate the pathogenic phenotypes appear to be necessary. Considerable evidence indicates that heavy alcohol and/or tobacco use increases the risk of optic neuropathy in LHON families (Smith PR et. al., Q J Med 1993;86:657-660, Chalmers RM et. al., Brain 1996;119:1481-1486 and Tsao K et. al., Br J Ophthalmol 1999;83:577-581, the contents of the cited reference are herein incorporated by reference), although one study did not find this association. Possible secondary genetic interactions are complex and not firmly established (Kerrison JB et. al., Am J Ophthalmol 2000;130:803-812, the contents of the cited reference are herein incorporated by reference).

Oxidative stress has been implicated in many disorders associated with mutations of mtDNA. A recent

investigation in an animal model identified reactive oxygen species (ROS) as a likely factor in the pathogenesis of LHON (Qi X et. al., Invest Ophthalmol Vis Sci 2003;44:1088-1096, the contents of the cited reference are herein  
5 incorporated by reference). Additionally, the mtDNA LHON pathogenic mutations were found to predispose cells to Fas-dependent apoptotic death *in vitro* (Danielson SR et. al., J Biol Chem 2002;277:5810-5815, the contents of the cited  
10 reference are herein incorporated by reference). These findings implied that there must be some nuclear modifier genes involved for developing LHON.

#### SUMMARY OF THE INVENTION

The inventor has revealed that some known and unknown SNPs are linked to onset of optic neuropathy including  
15 glaucoma and Leber's disease and completed the instant invention.

Accordingly, the present invention provides a set of genetic polymorphisms being associated with optic neuropathy, which comprises at least one polymorphism  
20 selected from the group consisting of:

- (1) AAG to AAT substitution at codon 198 of the Endothelin-1 gene (Lys198Asn);
- (2) -1370T>G polymorphism of the Endothelin-1 gene promoter region;

- (3) A138 insertion/deletion(A138I/D) polymorphism in exon 1 of the Endothelin-1 gene;
- (4) +70C>G polymorphism in 3' non-coding region of the Endothelin receptor A gene;
- 5 (5) +1222C>T polymorphism of the Endothelin Receptor A gene;
- (6) CAC to CAT substitution at codon 323 in exon 6 of the Endothelin Receptor A gene (His323His);
- (7) -231A>G polymorphism of the Endothelin Receptor A gene promoter region;
- 10 (8) CTG to CTA substitution at codon 277 in exon 4 of the Endothelin receptor B gene;
- (9) 9099C>A polymorphism of the Mitochondrial gene;
- (10) 9101T>G polymorphism of the Mitochondrial gene;
- 15 (11) 9101T>C polymorphism of the Mitochondrial gene;
- (12) 9804G>A polymorphism of the Mitochondrial gene;
- (13) 11778G>A polymorphism of the Mitochondrial gene;
- (14) -713T>G polymorphism of the Angiotensin II type 1 receptor gene promoter region;
- 20 (16) 3123C>A polymorphism of the Angiotensin II type 2 receptor gene;
- (25) CAA to CGA substitution at codon 192 of the Paraoxonase 1 gene (Gln192Arg);
- (26) TTG to ATG substitution at codon 55 of the Paraoxonase



- 1 gene (Leu55Met);
- (27) CGG to CAG substitution at codon 144 of the Noelin 2 gene (Arg144Gln);
- (32) GGA to CGA substitution at codon 389 of the  $\beta$ 1
- 5 adrenergic receptor gene (Gly389Arg);
- (35) 1105T>C polymorphism of the Myocilin gene (Phe369Leu);
- (36) 412G>A polymorphism of the Optineurin gene;
- (37) 1402C>T polymorphism of the E-Selectin gene;
- (38) The combination of polymorphisms of -857C>T of the
- 10 Tumor necrosis factor  $\alpha$  gene promoter region and 412G>A of the Optineurin gene;
- (39) The combination of polymorphisms of -863C>A of the Tumor necrosis factor  $\alpha$  gene promoter region and 603T>A of the Optineurin gene
- 15 (40) CGC to CCC substitution at codon 72 of the TP53 gene (Arg72Pro);
- (41) TAC to CAC substitution at codon 113 of the Microsomal epoxide hydrolase 1 gene (Tyr113His);
- (42) -110A>C polymorphism of the Heatshock protein 70-1
- 20 gene promoter region;
- (43) -338C>A polymorphism of the Endothelin converting enzyme gene promoter region;
- (44) -670A>G polymorphism of the CD95 gene promoter region;
- (45) AAG to AAA substitution at codon 119 of the Microsomal

epoxide hydrase 1 gene(Lys119Lys);

(47) GGA to AGA substitution at codon 16 of the  $\beta 2$  adrenergic receptor gene (Gly16Arg); and

(48) CAA to GAA substitution at codon 27 of the  $\beta 2$   
5 adrenergic receptor gene (Gln27Glu).

In addition, the present invention also provides a method for diagnosing or predicting susceptibility to optic neuropathy in a human subject, which comprising the steps of:

- 10       i) obtaining a biological sample from the subject,  
          ii) determining genotype of the sample in respect of the set of the polymorphisms defined as above, and  
          iii) diagnosing or predicting susceptibility to optic neuropathy in the subject based on the genotype.

15       According to the present invention, the optic neuropathy may preferably be glaucoma or Laber's disease. The polymorphism (1)-(39) and (42)-(48) may be used especially for glaucoma. Among them, those (1), (2), (5)-(7), (16), (26), (32), (43) and (45) may be used especially  
20       for normal tension glaucoma and those (4), (14), (25), (35), (36), (38), (42), (44), (47)-(48) may be used especially for primary open angle glaucoma. The polymorphisms (40) and (41) may be used especially for Laber's disease.

According to the present invention, the set of

polymorphisms may further comprise at least one other polymorphism which has been known to be associated with optic neuropathy.

5 In another aspect of the present invention, a kit for diagnosing or predicting susceptibility to optic neuropathy in a human subject which comprises primer set and/or probe suitable for determining genotype in respect of the set of genetic polymorphisms defined as above.

10 In further aspect of the present invention, newly identified SNPs are provided in Mitochondrial gene, Myocilin gene and Noelin 2 gene. Accordingly, the present invention encompass nucleotide fragment covering those SNPs. In general, in order to determine genotype in respect of said SNP, 90 or more contiguous nucleotide sequence  
15 containing the SNP may be required. Namely, an isolated polynucleotide consisting of a segment of the sequence:

8881 tctaagatta aaaatgcctt agccacttc ttaccacaag gcacacctac accccttacc  
8941 cccatactag ttattatcga aaccatcagc ctactcattc aaccaatagc cctggccgta  
9001 cgcctaaccg ctaacattac tgcaggccac ctactcatgc acctaattgg aagcgccacc  
20 9061 ctagcaatat caaccattaa ccttcctctt acacttatga tcttcacaat tctaattcta  
9121 ctgactatcc tagaaatcgc tgtgcctta atccaagcct acgttttacc acttctagta  
9181 agcctctacc tgcagcacia cacataatga cccaaccaac acatgcctat  
catatagtaa(SEQ ID NO:1)

wherein the segment comprises at least 90 contiguous nucleotide, and the at least 90 contiguous nucleotide includes position 9099 of the sequence, and wherein position 9099 of the sequence is A or an isolated

5 polynucleotide which is entirely complementary to the above segment; or

wherein the segment comprises at least 90 contiguous nucleotide, and the at least 90 contiguous nucleotide includes position 9101 of the sequence, and wherein  
10 position 9101 of the sequence is G; or  
an isolated polynucleotide which is entirely complementary to either of the above segment.

The present invention further provides an isolated polynucleotide consisting of a segment of the sequence:

15 301 actggaagc acgggtgctg tgggtgactc ggggagcctc tatttcagg gcgctgagtc  
361 cagaactgtc ataagatatg agctgaatac cgagacagtg aaggctgaga aggaaatccc  
421 tggagctggc taccacggac agttcccgta ttctgggggt ggctacacgg acattgactc  
481 ggctgtggat gaagcaggcc tctgggtcat ttacagcacc gatgaggcca aaggtgccat  
541 tgtctctccc aaactgaacc cagagaatct ggaactcgaa caaacctggg  
20 agacaaacat(SEQ ID NO:2)

wherein the segment comprises at least 90 contiguous nucleotide, and the at least 90 contiguous nucleotide includes codon 369, which is corresponding to the underlined nucleotides of the sequence, and wherein codon

369 is substituted such that it codes for Leu, or an isolated polynucleotide which is entirely complementary to the above segment.

The present invention further provides an isolated polynucleotide consisting of a segment of the sequence:

79741 ttagttocta caatggagtc atgtctggga agaatctagg gtccaatatg agcoacatgt  
79801 caaggggccag gtgtgcatca aagacaaagg gtgaagttat gagtcagagg ttggagtc  
79861 gtctgggtca aaggccagggt gtcagggttg gccatggttc catcttgatg cacaggagct  
10 79921 gaaggacagg atgacggaac tgttgccct gagctcggtc ctggagcagt acaaggcaga  
79981 cacggggacc attgtacgt tgcgggagga ggtgaggaat ctctccggca gtctggcggc  
80041 cattcaggag gagatgggtg cctacgggta tgaggacctg cagcaacggg tgatggccct  
80101 ggaggcccggt ctccagcct gcgccagaa gctgggtatg ccttgccct tgaccctgac  
80161 cctgatctc tgactgccac acccaactcc agtatcacct gttgtgcct agaagctgga  
15 80221 cacagttttg acctctaact tttaaccctc aacccttgac ctctctacct aaggctacac

(SEQ ID NO:3)

wherein the segment comprises at least 90 contiguous nucleotide, and the at least 90 contiguous nucleotide includes codon 144, which is corresponding to the underlined nucleotides of the sequence, and wherein codon 20 144 is substituted such that it codes for Gln, or an isolated polynucleotide which is entirely complementary to the above segment.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents correlation of clinical Characteristics of NTG Patients with AT2R 3123C>A Polymorphism and ACE I/D Polymorphism

Fig. 2 represents DHPLC tracing patterns in the Exon3C  
5 of the MYOC gene.

Fig. 3 represents novel missense mutation, Phe369Leu detected in exon 3 of the MYOC gene. Sequencing data depicting the mutation is shown (SEQ ID NO: 210).

Fig. 4 represents a DHPLC tracing of MYOC gene from a  
10 patient with POAG.

Fig. 5A represents the IOP after oral candesartan cilexetil or placebo.

Fig. 5B represents the ocular perfusion pressure after oral candesartan cilexetil or placebo

Fig. 5C represents the IOP after oral candesartan  
15 cilexetil in each of the 15 subjects.

#### PREFERRED EMBODIMENT OF THE INVENTION

In the present specification and claims, "genetic polymorphism" means genomic diversity between individuals  
20 at a locus. Genetic polymorphism may be single nucleotide substitution called as "Single nucleotide polymorphisms" or "SNPs" as well as those consisting of plural nucleotides. The genetic polymorphism may or may not be those affect on the phenotype of the individual. In addition, a nucleotide

sequence of an individual is different from the corresponding wild type sequence, i.e., having insertion, deletion or substitution on the wild type sequence, said nucleotidesequene is called as "genetic mutant" and the  
5 genetic mutant is also included in "polymorphic variant" according to the present invention.

In the present specification and claims, expression like "9099C>A" or "C9099A" means that the gene has a polymorphsm at position 9099, that is, there are two  
10 alleles of the gene and the one has cytosine or C and the other has adenine or A at 9099 (bi-allelic). It does not necessarily mean the frequent allele has C whereas the rare allele has A at said position.

The expression like "Gln192Arg" represents an amino  
15 acid substitution due to the base substitution in the gene coding for the amino acid sequence. For example, Gln192Arg represents Glycine at codon 192, i.e. amino acid number 192, is replaced with Arginine or Arg. This also means that there are polymorphic variants of the protein wherein the  
20 amino acid at codon 192 is Gln or Arg.

According to the present invention, determining genotype in respect of the genetic polymorphisms may be carried out by every single polymorphism, or plurality or all polymorphisms may be determined at the same time.

In the present invention, the method for diagnosing or predicting susceptibility to optic neuropathy in a human subject which comprises determining genotype in respect of the set of genetic polymorphism of which relationship with optic neuropathy is newly reported in this application. In addition to the genetic polymorphism identified as being linked to optic neuropathy by the instant invention, any other polymorphism which had been revealed as being linked to optic neuropathy may be detected together. By employing plural genetic polymorphisms linked to optic neuropathy, the diagnostic probability can be improved.

According to the present invention, the method used for determining genotype in respect of the genetic polymorphisms is not limited and may be any of those known to the art. Representative method for determining genotype in respect of the genetic polymorphisms include polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis, polymerase chain reaction followed by single strand conformation polymorphism (PCR-SSCP) analysis, ASO hybridization analysis, direct sequencing analysis, ARMS analysis, DGGE analysis, RNaseA cleaving analysis, chemical restriction analysis, DPL analysis, TaqMan® PCR analysis, Invader® assay, MALDI-TOF/MS analysis, TDI analysis, single nucleotide extension assay, WAVE assay,



one molecular fluorescent detection assay. According to the present invention, the detection method may be one of those or combination of two or more.

According to the present invention, biological sample  
5 to be used for detecting the genetic polymorphism is not specifically limited and may be hair, blood, saliva, lymph fluid, respiratory tract mucosa, cultured cells and urine.

In the specification and claims, "diagnosing or predicting susceptibility to optic neuropathy" includes not  
10 only diagnosing onset of optic neuropathy but also determining risk factors which hasten onset of the disease as well as accelerate the disease progresses.

According to the present invention, kits for detecting the genetic polymorphism as well as protein polymorphism  
15 identified as above are also provided. Said kits may comprise primers and/or probes which are specifically designed for detecting the above-identified genetic polymorphisms; antibodies for detecting the above-identified protein polymorphism. According to the present  
20 invention, said kit may be used for diagnosing or predicting susceptibility to optic neuropathy.

In the present specification and claims, the term "primer" denotes a specific oligonucleotide sequence which is complementary to a part of the target nucleotide

sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by either DNA polymerase, RNA polymerase or reverse transcriptase.

5       The term "probe" denotes a defined nucleic acid segment which can be used to identify a specific polynucleotide sequence present in samples or confirming target DNA or RNA in a gene modifying process, said nucleic acid segment comprising a nucleotide sequence complementary  
10      to the specific polynucleotide sequence to be identified.

          According to the present invention, primers and probes may be designed based on the targeted sequence so that they are specific to the position at which the targeted polymorphism is expected and/or surrounding sequence of the  
15      position so long as they are not identical to some other genes, i.e. it is necessary not to be repeating sequence nor palindrome sequence.

          According to the present invention, genetic polymorphisms which are linked to optic neuropathy,  
20      especially glaucoma and Leber's disease are identified. Based on the findings, the genotype in respect of the genetic polymorphisms of a biological sample obtained from an individual is determined and based on thus obtained genotype, onset of the disease or predicted risk for onset

of the disease can be determined.

In addition to the polymorphisms identified (1)-(48) as above, genotypes in respect of some other genetic polymorphisms which had been known to the art being highly  
5 associated with optic neuropathy may be determined for improved reliability of the diagnosis or prediction.

For example, two types of genetic polymorphisms in myocilin as well as optineurin genes have been revealed by the inventor to be associated with onset of primary open-  
10 angle glaucoma. In addition to the two genes, 4 other genetic polymorphisms including mutations had been identified to be associated with primary open-angle glaucoma. Almost 100% of the subjects having both the risk genotype in respect of the genetic polymorphisms of the  
15 present invention and of those already known to the art may develop glaucoma. That is, the set of the genetic polymorphisms will be useful for precrinical test.

In regard of some SNPS, the inventor confirmed correlation with optic neuropathy in a specific group, such  
20 as race or sex. Accordingly, said SNPs may preferably be used for diagnosing or predicting the risk for optic neuropathy in the specified group.

Further, statcal analysis of the genotyp in respect of the set of polymorphisms may provide useful information

such as predictive age of onset, predictive association with lifestyle-related diseases, predictive association with symptom factors. In addition, effect of some medical treatments may also be predictable based on the information.

5       According to the present invention, predicting susceptibility to optic neuropathy can be carried out before onset of the disease based on the genotype, and the subject can receive advice on how to remove the risk factor, for example, to improve life style or alter the environment. 10 In addition, it may possible to receive an early treatment such as reduction of the risk gene. an appropriate treatment can be started earlier. Consequently, those "order made treatment" can reduce the risk for vision loss.

For example, in case a subject has the genotype 15 linked to high risk for onset of optic neuropathy, inhibition of onset, reduction of the risk of onset or relief of symptoms can be expected by introducing to the subject the genotype linked to low risk for onset and expressing the same. Further, anti sense to the mRNA of 20 the allele of high risk for onset of optic neuropathy or RNAi method may be used for inhibiting expression of the high risk allele.

In another aspect, based on the genotype determination in respect of the set of polymorphisms shown in the present

invention, genetic etiology of optic neuropathy may be revealed and thus obtained etiology may be useful for development of novel medical agents.

Further, by combining genotype information which is associated with optic neuropathy obtained by the present invention and the other genotype information which is associated with life style diseases and the like, comprehensive risk for age-related, life-style related diseases can be predicted and used for high quality of life.

The present invention will be further illustrated by means of the examples shown below. It is to be expressly understood, however, that the examples are for purpose of illustration only and is not intended to limit of the scope of the invention.

**EXAMPLE 1 Genetic Variants of TP53 and EPHX1 in Leber's Hereditary Optic Neuropathy and their Relationship to Age at Onset**

**Purpose:** To determine whether genetic polymorphisms of the genes for oxidative stress and apoptosis cause the clinical variability in patients with Leber's hereditary optic neuropathy (LHON).

## MATERIALS AND METHODS

### Patients

We studied 86 unrelated Japanese patients with LHON carrying the 11778 mutation with homoplasmy. Their mtDNA  
5 mutation was confirmed by polymerase chain reaction followed by a restriction-enzyme assay which revealed concordant gain of the MaeIII site (Mashima Y et. al., Curr Eye Res 1998;17:403-408, the contents of the cited  
reference are herein incorporated by reference).

10 The mean age at the onset of visual loss in 86 LHON patients was  $25.1 \pm 13.0$  years with a range 3 to 65 years.

### Genomic DNA Extraction and Genotyping

DNA was extracted from peripheral blood leukocytes by the SDS-proteinase K and phenol/chloroform extraction  
15 method. Polymorphisms were examined in the oxidative stress-related gene, microsomal epoxide hydrolase (EPHX1) (Kimura K et. al., Am J Ophthalmol 2000;130:769-773, the contents of the cited reference are herein incorporated by  
reference).), and the apoptosis-related gene, Arg72Pro in  
20 TP53 (Ara S et. al., Nucleic Acids Res 1990;18:4961, the contents of the cited reference are herein incorporated by  
reference).

Each polymorphism was identified using polymerase chain reaction-restriction fragment length polymorphism

(PCR-RFLP) techniques (Table 1).

Table 1. Primer sequences, product size, and annealing temperatures

Gene	Primer sequences		Product Size (bp)	Annealing Temperature (°C)	Restriction Enzyme	
TF53	F	TTG CCG TCC CAA GCA ATG GAT GA	199	60.0	Acc II	(SEQ ID NO:4)
	R	TCT GGG AAG GGA CAG AAG ATG AC				(SEQ ID NO:5)
EPHX1	F	GAT CGA TAA GTT CCG TTT CAC C	165	56.0	EcoR V	(SEQ ID NO:6)
	R	TCA ATC TTA GTC TTG AAG TGA GGA T				(SEQ ID NO:7)

## RESULTS

The associations between age at onset and the polymorphisms were presented in Table 2-1 and Table 2-2.

5 Table 2-1. Association between age at onset and TP53 (Arg72Pro) and EPHX1 (Tyr113His) gene polymorphism in Leber's hereditary optic neuropathy

Gene	Genotype		P
	Arg/Arg	Arg/Pro + Pro/Pro	
TP53 (Arg72Pro)			
Age at onset	20.7±10.6 (n=35)	28.1±13.8 (n=51)	0.009
EPHX1 (Tyr113His)			
Age at onset	27.9±13.9 (n=45)	22.1±11.4 (n=41)	0.038

P Value for t-test

10 Table 2-2. Association between age at onset and TP53 (Arg/Arg) and EPHX1 (His/His) gene polymorphism in Leber's hereditary optic neuropathy

Group 1	Group 2	Group 3	p
Arg/Arg and His/His	Arg/Arg or His/His	others	
17.7±9.3 (n=19)	25.3±11.3 (n=38)	29.8±15.1 (n=29)	0.0044

P value for Kruskal-Wallis

- Group 1: Patients who have Arg/Arg at codon 72 in TP53 and His/His at codon 113 in EPHX1
- 15 Group 2: Patients who have Arg/Arg at codon 72 in TP53 but not His/His at codon 113 in EPHX1, or His/His at codon 113 in EPHX1 but not Arg/Arg at codon 72 in TP53
- Group 3: Patients other than Groups 1 and 2

20 As shown in Table 2-1, the codon 72 genotype in TP53



and the codon 113 genotype in EPHX1 were significantly associated with younger age at onset of Leber's hereditary optic neuropathy.

As shown in Table 2-2, the co-existence of the Codon  
5 72 genotype in TP53 and the codon 113 genotype in EPHX1 were significantly associated with younger age at onset of Leber's hereditary optic neuropathy.

These results indicated that detection of the Arg/Arg homozygote in TP53 and His/His homozygote in EPHX1  
10 make possible the early diagnosis and early treatment of Leber's hereditary optic neuropathy.

These results also indicated that the Codon 72 polymorphism may interact with mitochondrial dysfunction to influence disease expression. Individual variations may  
15 exist in the apoptotic response that is correlated with the polymorphism at codon 72 of p53. Bonafe et al (Biochem Biophys Res Commun 2002;299:539-541.). reported that cultured cells from healthy subjects carrying the Arg/Arg genotype underwent more extensive apoptosis than cells from  
20 Arg/Pro subjects in response to the cytotoxic drug cytosine arabinoside. Thus, naturally occurring genetic variability at the p53 gene could partly explain individual differences in *in vivo* susceptibility of cells to a chemotherapeutic drug. Dumount et al (Nat Genet 2003;33:357-365). reported

that the Arg72 variant was more efficient than the Pro72 variant at inducing apoptosis, with at least one mechanism underlying this greater efficiency being enhanced localization of Arg72 variant to mitochondria in tumor  
 5 cells. The synthetic p53 inhibitors might be highly effective in treating LHON in which neurons died by apoptosis triggered by mitochondrial impairment and oxidative stress.

Partial nucleotide sequences for EPHX1 and TP53 genes containing the targeted polymorphism are as follows:

EPHX1 Tyr113His Codon 113 (underlined) (TAC to CAC change)

181 tgctgggctt tgccatctac tggttcatct cccgggacaa agaggaaact ttgccacttg  
 241 aagatgggtg gtgggggcca ggcacgaggt ccgcagccag ggaggacagc agcatccgcc  
 301 ctttcaaggt ggaaacgtca gatgaggaga tccacgactt acaccagagg atcgataagt  
 15 361 tccgtttcac cccacctttg gaggacagct gcttccacta tggcttcaac tccaactacc  
 421 tgaagaaagt catctcctac tggcgggaatg aatttgactg gaagaagcag gtggagattc  
 481 tcaacagata ccctcacttc aagactaaaa ttgaagggtt ggacatccac ttcattccag  
 541 tgaagccccc ccagctgccc gcaggccata ccccgagccc cttgctgatg gtgaacggct  
 601 ggccccgctc ttctctacgag ttttataaga tcattccact cctgactgac cccaagaacc  
 20 661 atggcctgag cgatgagcac gtttttgaag tcattcgccc ttccatccct ggctatggct  
 721 tctcagaggc atcctccaag aagggggttca actcgggtggc caccgccagg  
 atctttttaca (SEQ ID NO:8)

TP 53 Codon 72 (underlined): CGC (Arg) to CCC (Pro),

25 13081 gcaggccac caccgccacc ccaacccag cccctagca gagacctgtg ggaagcgaaa

13141 attccatggg actgactttc tgctctgtc tttcagactt cctgaaaaca acgttctggt  
 13201 aaggacaagg gttgggctgg ggaactggag ggctggggac ctggagggt ggggggctgg  
 13261 ggggctgagg acctggctct ctgactgtc tttcaccca tctacagtcc cccttgccgt  
 13321 **cccaagaat** **ggatgattg** atgctgtccc cggacgatat tgaacaatgg ttoactgaag  
 5 13381 acccaggtcc agatgaagct ccagagaatgc cagaggctgc **tcccgcgtg** gccctgcac  
 13441 cagcagctcc tacaccggcg gccctgcac cagcccccct ctggcccctg **tcatattctg**  
 13501 **tccattccca** gaaaacctac cagggcagct acggtttccg totgggcttc ttgoattctg  
 13561 ggacagccaa gtctgtgact tgcacggtca gttgccctga ggggctggct tccatgagac  
 13621 ttcaatgctt gccgctatcc cctgcattt cttttgttg gaactttggg attcctcttc  
 10 13681 accctttggc ttctgtcag tgttttttta tagtttacc acttaatgtg tgatctctga  
 13741 ctctctgccc aaagttgaat attccccct tgaatttggg cttttatcca tcccatcaca  
 13801 cctcagoat ctctctggg gatgcagaac tttttttt cttcatccac gtgtattcct(BBQ ID  
NO:9)

- 15 **Example 2 Mitochondrial DNA mutations related with Leber's hereditary optic neuropathy in primary open-angle glaucoma and normal-tension glaucoma**

## **Materials and Methods**

### 20 **Patients**

A total of 651 blood samples were collected at seven institutions in Japan. There were 201 POAG patients, 232 NTG patients, and 218 normal controls, and none of the subjects was related to others in this study.

The mean age at the time of examination was  $61.2 \pm 16.0$  years in POAG,  $58.8 \pm 13.6$  years in NTG, and  $70.6 \pm 10.9$  years in the control subjects. The mean age of the control subjects was significantly older than that of POAG patients ( $P < 0.001$ ) and the NTG patients ( $P < 0.001$ ). We purposely selected older control subjects to reduce the probability that a subset of them would eventually develop glaucoma. There were 112 (55.7%) men in the POAG group, 108 (46.6%) in the NTG group, and 89 (40.8%) in the control group.

Patients were considered to have POAG if they had a normal open-angle, a cup-disc ratio greater than 0.7 with typical glaucomatous visual field loss on either Goldmann or Humphrey perimetry, and the absence of ocular, rhinologic, neurologic, or systemic disorders which might be responsible for the optic nerve damage. Patients with NTG had an IOP of 21 mmHg or lower. Patients with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded.

Two-hundred-eighteen control samples were obtained from Japanese subjects who had no known eye abnormalities except for cataracts. These subjects were older than 40 years, had IOPs below 21 mm Hg, had normal optic discs, and no family history of glaucoma.

**Detection of mtDNA Mutations by Invader® Assay**

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods of phenol-chloroform extraction.

5           The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the six mtDNA mutations (G3460A, T9101C, G9804A, G11778A, T14484C, and T14498C) by the Invader® assay are shown in Table 3.

10

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5

[illegible]

Invader® assay FRET-detection 256-well plates (Third Wave Technologies, Inc, Madison, WI) contains the generic components of an Invader® assay (Cleavease® enzyme VIII, FRET probes, MOPS buffer, and polyethylene glycol) dried in  
5 each of the individual wells. The biplex format of the Invader® assay enabled simultaneous detection of two DNA sequences in a single well.

The detail method was described previously. In brief,  
8 µl of the primary probe/Invader®/mixture and total DNA  
10 (10 ng) samples were added to each well of a 96-well plate, and were denatured by incubation at 95° C for 10 min. After 15 µl of mineral oil (Sigma, St. Louis, MO) was overlaid on all reaction wells, the plate was incubated isothermally at 63° C for 2 hours in a PTC-100 thermal cycler (MJ Research,  
15 Waltham, MA) and then kept at 4° C until fluorescence measurements. The fluorescence intensities were measured on a CytoFlour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation at 485 nm/20 nm (wavelength/ bandwidth) and emission at 530 nm/25 nm for  
20 FAM dye; excitation at 560 nm/20 nm and emission at 620 nm/40 nm for Redmond RED (RED) dye. Each samples was tested in duplicate in the same plate and two fluorescence measurements were performed in each plate. Thus, four measurements were obtained for each sample and they were

averaged.

#### Direct DNA Sequencing

To detect mutations by direct sequencing, the PCR products were first purified with the QIAquick PCR  
 5 Purification Kit (QIAGEN, Valenica, CA, USA) to remove unreacted primers and precursors. The sequencing reactions were then performed using the ABI PRISM BigDye Terminator (v.3.1) Cycle Sequencing Kit, according to the  
 manufacturer's protocol (Applied Biosystems). The data were  
 10 collected by the ABI PRISM 310 Genetic Analyzer and analyzed by the ABI PRISM sequencing analysis program (v.3.7).

Table 4. Primer sequences

mutation		Primer Sequences (5' to 3')	
3460	F	CAG TCA GAG GTT CAA TTC CTC	<u>(SEQ ID NO:28)</u>
	R	TGG GGA GGG GGG TTC ATA GTA	<u>(SEQ ID NO:29)</u>
11778	F	GGC GCA GTC ATT CTC ATA AT	<u>(SEQ ID NO:30)</u>
	R	AAG TAG GAG AGT GAT ATT TG	<u>(SEQ ID NO:31)</u>
14484	F	none	
	R	GCT TTG TTT CTG TTG AGT GT	<u>(SEQ ID NO:32)</u>
9101	F	AAA ATG CCC TAG CCC ACT TC	<u>(SEQ ID NO:33)</u>
	R	GTC ATT ATG TGT TGT CGT GC	<u>(SEQ ID NO:34)</u>
9804	F	CAC ATC CGT ATT ACT CGC AT	<u>(SEQ ID NO:35)</u>
	R	CGG ATG AAG CAG ATA GTG AG	<u>(SEQ ID NO:36)</u>

#### RESULTS

15 A total of 651 Japanese subjects were studied. When a nucleotide substitution is located within a primary probe



or an invader probe, the examined cases showed no reaction to both probes by Invader assay. In such cases, direct sequence analysis showed single nucleotide polymorphisms (SNPs) at the nucleotide position of 9099, 9101, 9102, 9797, and 9815.

As shown in Table 5, 7 patients including 5 females and 2 males harbored 5 mutations of mtDNA, and have not developed LHON. Two patients (Cases 1 and 2) harbored novel amino acid changes which have not been to associated with LHON, and 5 patients (Cases 3 to 7) harbored LHON mutations.

These mtDNA mutations were not detected in normal controls.

Table 5.

Case	mtDNA mutation	Patient
1	C9099A mutation (Ile to Met)	POAG (Male)
2	T9101G mutation (Ile to Ser)	POAG (Female)
3	T9101C mutation (Ile to Thr)	POAG (Female)
4	G9804A mutation (Ala to Thr)	POAG (Male)
5	G9804A mutation (Ala to Thr)	NTG (Female)
6	G11778A mutation (Arg to His) heteroplasmy 80%	POAG (Female)
7	G11778A mutation (Arg to His) heteroplasmy 15%	NTG (Male)

As described above, we found 5 mtDNA mutations including 2 novel mtDNA mutations in glaucoma patients. These results indicated that mtDNA mutations is one of the risk factor to develop or progress the glaucoma, and detection of the mtDNA mutations makes possible the early diagnosis and early treatment of glaucoma.

Partial nucleotide sequences of mitochondrial gene containing the targeted mutations/polymorphism are as follows:

5 C9099A, T9101G (underlined)

8881 tctaagatta aaaatgccct agcccacttc ttaccacaag gcacacctac accccttacc  
8941 ccctactag ttattatcga aaccatcagc ctactcattc aaccaatagc cctggccgta  
9001 cgctaaccg ctaacattac tgcaggccac ctactcatgc acctaattgg aagcgccacc  
9061 ctagcaatat caaccattaa ccttccctct acacttatga tcttcacaat tctaattcta  
10 9121 ctgactatcc tagaaatcgc tgtcgcctta atccaagcct acgttttccac acttctagta  
9181 agcctctacc tgcacgacaa cacataatga cccaccaatc acatgcctat catatagtaa  
(SEQ ID NO:37)

G9804A (underlined)

15 9541 taggagggca ctggcccccacacaggcatca ccccgctaaa tcccctagaa gtcccactcc  
9601 taaacacatc cgtattactc gcctcaggag tatcaatcac ctgagctcac catagtctaa  
9661 tagaaaaacaa ccgaaaaccaa ataattcaag cactgcttat tacaatttta ctgggtctct  
9721 attttaccct cctacaagcc tcagagtact tcgagtctcc cttcaccatt tccgacggca  
9781 tctacggctc aacatttttt gtagccacag gcttcacagg acttcacgic attattggct  
20 9841 caactttcct cactatctgc ttcacccgcc aactaatatt tcaatttaca tccaacatc  
9901 actttggctt cgaagccgcc gctgatact ggcatthtgt agatgtgggt tgactatttc  
(SEQ ID NO:38)

G11778A (underlined)

25 11641 agccctcgta gtaacagcca ttctcatca aaccctctga agcttcaccg gcgcagtcac

11701 tctcataatc gccacgggc ttacatctc attactattc tgcttagcaa actcaaaacta  
 11761 cgaacgcact cacagtcgca tcataatcct ctctcaagga cttcaaaactc tactccact  
 11821 aatagctttt tgatgacttc tagcaagcct cgctaacctc gccttacctc ccactattaa  
 11881 cctactggga gaactctctg tgctagtaac cagttctcc tgatcaaata tcactctcct  
 5 11941 acttacagga ctcaacatac tagtcacagc cotatactcc ctctacatat ttaccacaac  
 12001 acaatggggc tcactcacc accacattaa caacataaaa ccctcattca cagagaaaa  
(SEQ ID NO:39)

10 **Example 3 Gene polymorphisms of the renin-angiotensin  
 aldosterone system associate with risk for developing  
 primary open-angle glaucoma and normal-tension glaucoma**

**Purpose:** Multiple environmental and genetic factors may be  
 involved in pathogenesis of glaucoma. To predict genetic  
 15 risk of glaucoma, an association study in gene  
 polymorphisms of the renin-angiotensin-aldosterone (R-A-A)  
 system was performed.

#### **MATERIALS and METHODS**

##### **Patients and Control study subjects**

20 A total of 551 blood samples were collected at seven  
 institutes in Japan. They were 162 POAG patients, 193 NTG  
 patients, and 196 normal subjects, and none of the subjects  
 was related to others in this study.

The average age at examination was  $58.8 \pm 13.7$  years

in NTG,  $62.0 \pm 15.4$  years in POAG, and  $71.2 \pm 10.4$  years in normal subjects. The average age of the normal control subjects is significantly higher than NTG patients ( $p < 0.001$ ) or POAG patients ( $p < 0.001$ ), respectively. This  
5 could reduce the possibility that a subset will eventually develop glaucoma. The familial history was recorded in 66 (34.2%) out of 127 NTG patients and 49 (30.2%) out of 113 POAG patients. Male patients were 89 (46.1%) in NTG and 87 (53.7%) in POAG, and 77 (39.3%) in normal subjects.

10 One hundred ninety-six Japanese control samples were obtained from individuals who had no known eye abnormalities except cataract. These subjects were older than 40 years with IOP below 21 mmHg, no glaucomatous disc change, and no family history of glaucoma.

#### 15 Genotyping

Seven genes and 10 polymorphisms in the R-A-A system were determined for each subject with glaucoma or normal Japanese control with renin (REN) I8-83G>A (Frossard PM et. al., Hypertens Res 1998;21:221-225, the contents of the  
20 cited reference are herein incorporated by reference), angiotensin II type 1 receptor (AT1R) 1166A>C, -521C>T, -713T>G (Nalogowska-Glosnicka K et. al., Med Sci Monit 2000;6:523-529 and Erdmann J et. al., Ann Hum Genet 1999;63:369-374, the contents of the cited reference are

herein incorporated by reference), angiotensin II type 2  
receptor (AT2R) 3123C>A (Katsuya T et. al., Mol Cell  
Endocrinol 1997;127:221-228, the contents of the cited  
reference are herein incorporated by reference), cytochrome  
5 P45011B1 (CYP11B1) -344T>C (Tsujiya Y et. al., Hypertens  
Res 2001;24:105-109, the contents of the cited reference  
are herein incorporated by reference), and chymase (CYM)  
3123C>A, were identified using by polymerase chain  
reaction-restriction fragment length polymorphism (PCR-  
10 RFLP). The angiotensin-converting enzyme (ACE)  
insertion/deletion (I/D) was determined only by PCR and  
agarose gel electrophoresis. To avoid the false  
determination of ACE/ID polymorphism, I allele specific  
amplification was carried out following the protocol of  
15 Lindpaintner et al (N Engl J Med 1995; 332: 706-711, the  
contents of the cited reference are herein incorporated by  
reference). Genomic DNA was isolated from peripheral blood  
lymphocytes by phenol-chloroform extraction. The primer  
sets and restriction enzymes used were listed in Table 6.

Table 6. Primer pair sequences used for PCR amplification and restriction enzymes of polymorphic sites in renin angiotensin system

Gene	polymorphism	Primer sequences	Annealing temp	Product size	Restriction enzyme	Digested products
REN	18-83bpA	TGAGGTCGAGTCGCGGCGGCT TCCGACAGAGCGGCGACGCT	68°C	230bp	Mbo I	0: 250bp A: 171+79bp (SEQ ID NO:41)
	I/D 1st step	GATCGGATGATGTCGACGAT GATGATCTCTGCGGCGGCTGTC	63°C	D: 319bp I: 597bp		(SEQ ID NO:42)
ACE	2nd step	TCCGACGACGCGGCGGCGGCTG TCCGCGGCGGCTGCGGTCGCGGAC	67°C	D/D: no products I: 335bp		(SEQ ID NO:44)
	1166D-C	GAAGTGGAGTACGATGTCGAC GATGTCGATGTCGACGATGTC	60°C	233bp	Dde I	A: 253bp (SEQ ID NO:46)
AGT	-521C-T	CGATGCTGATGATGATGATG CGATGCTGATGATGATGATG	60°C	270bp	Ssp I	C: 270bp (SEQ ID NO:47)
	-717D-G	ATGCTGAGTGGACGATGATG ATGCTGAGTGGACGATGATG	55°C	232bp	Eco RI	T: 144+125bp (SEQ ID NO:48)
ACE	3123C-A	GATGTCGATGTCGTCGAC GATGTCGATGTCGTCGAC	55°C	340bp	Alu I	G: 340bp (SEQ ID NO:49)
	-340C-T	CGTGGAGGATGATGATGAC CTGGGAGGATGATGATGAC	63°C	404 bp	Bst III	A: 271+13bp C: 33bp + 71bp (SEQ ID NO:50)
CYP11B1	-340C-T	CGTGGAGGATGATGATGAC CTGGGAGGATGATGATGAC	63°C	404 bp	Bst III	(SEQ ID NO:51)
OMA	-191A-G	ATCCCGGAGTGGGATGATGATG ATCCCGGAGTGGGATGATGATG	51°C	283bp	Bst XI	A: 283bp (SEQ ID NO:52)
					G: 191+90bp (SEQ ID NO:53)	(SEQ ID NO:57)

The genotyping angiotensinogen (AGT) T174M, M235T was determined using by Invader assay® (Lyamichev V et. al., Nat Biotechnol 1999;17:292-296, the contents of the cited reference are herein incorporated by reference).

5     **RESULTS**

Genotype distribution of R-A-A system in Japanese population

Of 10 polymorphisms in R-A-A system, two showed a significantly difference in frequencies of genotypes:  
10     AT1R/-713T>G for POAG, and AT2/3123C>A for NTG (Table 7). A 3123C>A polymorphism was associated with only female patients with NTG.

A frequency of homozygous G genotype (GG) in AT1R/-713T>G polymorphism was significantly higher ( $p=0.04$  for  
15     TT+TG v GG) in POAG patients (4.2%) than in controls (0.5%). A frequency of CA+AA genotypes in AT2R/3123C>A polymorphism was significantly higher ( $p=0.011$  for CC v CA+AA) in female patients with NTG (70.8%) than in female controls (55.0%).  
Table 7. Association between glaucoma (POAG and NTG) and  
20     gene polymorphism of the renin-angiotensin aldosterone system.

Gene	Gene Polymorphism	Genotype Frequency		p
		TT+TG	GG	
AT1	-713T>G	POAG (n=165)	158 (95.8%)	7 (4.2%)
		NTG (n=208)	208 (100%)	0 (0.0%)
		Control (n=198)	197 (99.5%)	1 (0.5%)
			CC	CA+AA
AT2	3123C>A (Female)	POAG (n=79)	34 (43.0%)	45 (56.0%)
		NTG (n=120)	35 (29.2%)	85 (70.8%)
		Control (n=111)	54 (45.0%)	66 (55.0%)

**Association between two promoter polymorphisms in AT1R in POAG patients**

A frequency of POAG carriers with combined  
 5 homozygous -521T and homozygous -713G (4.2%) was  
 significantly higher (p=0.011) than that of normals (0%)  
 (Table 8-1). Only POAG patients, neither NTG nor normal  
 subjects, had this genotype.

10 **Table 8-1. Distribution of genotypes of AT1R -521T allele  
 and -713G allele**

Group	A	B	p
POAG (n=165)	7 (4.2%)	158 (95.8%)	0.011
NTG (n=208)	0 (0.0%)	208 (100.0%)	
Control (N=198)	0 (0.0%)	198 (100.0%)	

A: Subjects with two -521 alleles and two -713G alleles

B: Subjects not satisfying the criteria for Group A.



These results indicated that gene polymorphism of the renin-angiotensin aldosterone system is one of important genetic risk factors for development of glaucoma. Detection of AT1R/-731T>G polymorphisms makes possible the early diagnosis and early treatment of POAG. Especially, specific genotype of combined homozygous -521T and homozygous -713G in the AT1R gene is useful for the early diagnosis of POAG. Detection of the AT2R/3123C>A polymorphisms make possible the early diagnosis and early treatment of female patient with NTG.

**Clinical Characteristics of NTG Patients with AT2R 3123C>A Polymorphism and ACE I/D Polymorphism**

The clinical features recorded in the glaucoma patients were age at diagnosis, untreated maximum IOP (defined as IOP at diagnosis), and visual field defects at the initial examination (defined as visual field defects at diagnosis. The severity of the visual field defects was scored from 1 to 5. Data obtained with different perimeters were combined using a five-point scale defined as follows:

1 = no alteration; 2 = early defect; 3 = moderate defect; 4 = severe defect; and 5 = light perception only or no vision. Field defects were judged to be early, moderate, or severe according to Kozaki's classification based on the results of Goldmann perimetry or the classification used for the

Humphrey field analyzer. The former classification is most widely used in Japan.

Significant association of the clinical characteristics of visual field score was detected between male glaucoma patients with AT2R genotype. Visual field score in male POAG patients with C genotype had worse than those with A genotype ( $P=0.04$ , Table 8-2). No significant association of the clinical characteristics (age, IOP, and visual field score) was detected between female glaucoma patients with C/C and those with C/A+A/A genotypes. The visual field score had a tendency to be worse in NTG patients with C/C genotype than those with C/A+A/A genotypes ( $P = 0.165$ ).

However, when combined with ACE insertion/deletion polymorphism, female patients with NTG who carried C/C in the AT2R gene as well as ID+DD in the ACE gene had significantly worse visual field scores than the other three combined genotypes ( $P = 0.012$ ; Table 8-3, Figure 1).

Table 8-2 Comparison of Clinical characteristics of male glaucoma patients according to AT2R genotypes

AT2 3123G>A

Male

Phenotype	Phenotype Variable	C	A	P value*
POAG	Age at diagnosis (ys)	57.0±10.9 (n=62)	56.9±14.0 (n=46)	0.808
	IOP at diagnosis (mm Hg)	26.8±6.7 (n=55)	27.5±6.7 (n=43)	0.522
	Visual field score at diagnosis	3.27±0.96 (n=62)	2.89±0.74 (n=46)	0.015

\* P value for logistic regression analysis

Table 8-3 Comparison of clinical characteristics of female patients  
with NTG according to ACE genotypes (Insertion/deletion) and AT2R  
genotypes (3123C>A)

Clinical characteristics	ACE			I/I			I/D+D/D			P
	AT2R	C/C	I/I	C/A + A/A	C/C	I/D+D/D	C/A + A/A	C/C	I/D+D/D	
Age at diagnosis (ys)	63.6±10.9 (n=15)	57.0±11.2 (n=47)	56.2±14.1 (n=23)	58.5±12.0 (n=51)	0.313					
IOP at diagnosis (mm Hg)	16.0±2.2 (n=16)	16.5±2.6 (n=43)	16.1±2.7 (n=20)	16.5±2.2 (n=49)	0.75					
Visual field score at diagnosis	2.47±0.51 (n=17)	2.64±0.53 (n=47)	3.13±0.76 (n=23)	2.65±0.59 (n=52)	0.012†					

\* P value tested by Kruskal-Wallis test

† P<0.05

Partial nucleotide sequences of AT1R and AT2R genes containing the targeted polymorphism are as follows:

AT1R -713(the underlined "t") T>G

1861 attactgtaa actacagtca cctactcac ctatctaaca ttaattgatt ttggttaaac  
5 1921 taatctaadc ttgctttctg gcatcaacct cacttgacca tgggtgatag tccctttcat  
1981 atgttattgg atTcaatttg cctacatttt gttgagaatt tttatctata ctcttaagaa  
2041 atattgatct gtagtctcgt gatgtcttta tctggttttg ttatcagggt gatactggcc  
2101 tcatagcatg agttggggaga tcatccttac tcttctattt ttggaagag tttgtgaaga  
2161 attgatatta tttcttcttt aaatatttat tgggttttta aaatacattt ttaaaatgca  
10 (SEQ ID NO:58)

AT2R 3123(the underlined "c") C>A, the underlined oligonucleotide sequences were used for primers

ggattcagatttctcttgaacacatgcttctgttctttagtgggttttatatccatttttatcaggatt  
tctctttgaaccagaaccagcttttcaactcattgcatcatttacaagacaacattgtaagagagatgag  
15 cacttctaagttgagtatattataatagattagtagtactggattattcaggctttaggcatatgcttcttta  
aaaaggctataaattatattctcttgcatttcacttgagtgagggtttatagtttaactataactacat  
attgaatagggttaggaatatagattaaatcatactcctatgc  
(SEQ ID NO:59)

(Based on GenBank accession No. AY536522, the AT2R 3123 corresponds  
20 nucleotide number 4926)

4741 gtgtttctta gtgggggttt atatccattt ttatcaggat ttcctcttga accagaacca  
4801 gtctttcaac tcaattgcat atttacaaga caacattgta agagagatga gcaacttctaa  
4861 gttgagtata ttataataga ttagtactgg attattcagg ctttaggcat atgcttcttt  
4921 aaaaggcta taaattatat tctcttgc tttcacttga gtggagggtt atagttaate  
25 4981 tataactaca tattgaatag ggctaggaat atagattaaa tcaactcct atgctttagc

5041 ttatttttac agttatagaa agcaagatgt actataacat agaattgcaa tctataatat  
5101 ttgtgtgttc actaaactct gaataagcac tttttaaaaa actttctact cattttaatg  
(SEQ ID NO:60)

- 5     **Example 4 Gene polymorphisms of the Endothelin gene**  
**associate with risk for developing normal-tension glaucoma**

#### **Methods**

#### **Patients**

- 10             A total of 605 blood samples were collected. There  
were 178 POAG patients, 214 NTG patients, and 213 normal  
controls, and none of the subjects was related to others in  
this study. Patients were considered to have POAG if they  
had a normal open-angle, a cup-disc ratio greater than 0.7  
15     with typical glaucomatous visual field loss on either  
Goldmann or Humphrey perimetry, and the absence of ocular,  
rhinologic, neurological or systemic disorders which might  
be responsible for the optic nerve damage. Patients with  
NTG had an IOP of 21 mmHg or lower. Patients with  
20     exfoliative glaucoma, pigmentary glaucoma, and  
corticosteroid-induced glaucoma were excluded. Control  
samples were obtained from Japanese subjects who had no  
known eye abnormalities except for cataracts. These  
subjects had IOPs below 21 mm Hg, had normal optic discs,

and no family history of glaucoma.

**Detection of G/T polymorphism of endothelin (ET) gene by  
Invader assay**

DNA was isolated from peripheral blood lymphocytes  
5 by standard methods of phenol-chloroform extraction, and  
G/T polymorphism (Lys/lys, Lys/Asn and Asn/Asn) at codon  
198 in exon 5 of ET gene was determined by the Invader®  
assay. The primary probes (wild and mutant probes) and  
Invader® oligonucleotides (Invader® probe) used to detect  
10 the G/T polymorphism of ET gene are shown in Table 9.

15

20

Table 9

Mutation	nucleotide change	Target	Probe	Sequence	T <sub>m</sub>	Dye	(SEQ ID NO: 63)
EDN Exs 6T	G to T	Sense	Wild	Flap sequence-CTTGGCTTTGAGCTTGG	64.6	FAM	(SEQ ID NO: 63)
			Mutant	Flap sequence-ATTGGCTTTGAGCTTGG	64.0	RED	(SEQ ID NO: 63)
			Invader	8TTGCGGTCGATTAAGCTCTCTGGAGGTT	78.9		(SEQ ID NO: 63)

5

Invader® assay FRET-detection 96-well plates (Third Wave Technologies, Inc, Madison, WI) contains the generic components of an Invader® assay (Cleavase® enzyme VIII,

FRET probes, MOPS buffer, and polyethylene glycol) dried in each of the individual wells. In brief, 8  $\mu$ l of the primary probe/Invader<sup>®</sup>/mixture and total DNA (10 ng) samples were added to each well of a 96-well plate, and were denatured by incubation at 95° C for 10 min. After 15  $\mu$ l of mineral oil (Sigma, St. Louis, MO) was overlaid on all reaction wells, the plate was incubated isothermally at 63° C for 2 hours in a PTC-100 thermal cycler (MJ Research, Waltham, MA) and then kept at 4° C until fluorescence measurements. The fluorescence intensities were measured on a CytoFluor 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation at 485 nm/20 nm (wavelength/ bandwidth) and emission at 530 nm/25 nm for FAM dye; excitation at 560 nm/20 nm and emission at 620 nm/40 nm for Redmond RED (RED) dye. Each sample was tested in duplicate in the same plate and two fluorescence measurements were performed in each plate. Thus, four measurements were obtained for each sample and they were averaged.

## Results

The genotype frequencies of G/T polymorphism (Lys/Lys, Lys/Asn and Asn/Asn) at codon 198 in exon 5 of ET gene are presented in Table 10.

Table 10. The genotype frequency at codon 198 in exon 5 of



## ET gene

Group	n	Genotype Frequency			p	Genotype Frequency		p
		Lys/Lys	Lys/Asn	Asn/Asn		Lys/Lys	Lys/Asn + Asn/Asn	
Control	213	94 (44.1%)	93 (43.7%)	26 (12.2%)		94 (44.1%)	119 (55.9%)	
NTG	214	120 (56.1%)	72 (33.6%)	22 (10.3%)	0.046	120 (56.1%)	94 (43.9%)	0.014
POAG	178	82 (46.1%)	77 (43.3%)	19 (10.7%)		82 (46.1%)	96 (53.9%)	

These results indicated that Lys/Lys homozygote of ET-1 gene at codon 198 in exon 5 is one of the risk factor to develop or progress the NTG, and detection of the Lys/Lys homozygote makes possible the early diagnosis and early treatment of NTG.

Partial sequence of EDN1 comprising codon 198 is as follows:

EDN1 Codon 198(underlined): aag (Lys) to aat (Asn)

9061 ttgaggtttt atcaaaagagt tgcggcgggt ggtgaaagtt cacaaccaga ttcaggtttt  
 9121 gtttgtgcc aatttctaatt ttacatgttt cttttgccaa agggtgattt ttttaaaata  
 9181 acatttggtt tctcttatct tgcctttatta ggtcggagac catgagaaac agcgctcaat  
 9241 catcttttca tgatcccaag ctgaaaggca agccctccag agagcggttat gtgaccacaca  
 9301 accgagcaca ttggtgacag accttcgggg cctgtctgaa gccatagcct ccacggagag  
 9361 ccctgtggcc gactctgcac tctccacctt ggctgggata agagcaggag catcctctgc  
 (SEQ ID NO:64)

(tga) is the translation termination codon)

Example 5 Novel MYOC Gene Mutation, Phe369Leu, in Japanese Patients with Primary Open-angle Glaucoma Detected by

## Denaturing High-performance Liquid Chromatography

**Purpose:** To screen for mutations in the *MYOC* gene in Japanese patients with primary open-angle glaucoma (POAG) using denaturing high-performance liquid chromatography (DHPLC).

### Materials and Methods

#### Patients

Blood samples were collected from 171 POAG patients and 100 normal subjects at seven Japanese medical institutions. The subjects were unrelated, and their mean age at the time of examination was  $55.1 \pm 16.0$  ( $\pm$  standard deviation) years for the patients with POAG and  $70.5 \pm 10.6$  years for the normal subjects. We purposely selected older control subjects to reduce the probability that a subset of them would develop glaucoma.

A detailed family history was obtained by interviews in 55 POAG patients (32.2%). There were 91 men (53.2%) in the POAG patients, and 41 men (41.0%) in the normal subjects.

#### DNA Extraction and PCR Conditions

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. The seven exonic regions of the *MYOC* gene were amplified by polymerase chain

reaction (PCR) using the primer sets listed in Table 11. For high-throughput analysis of the patients, samples from three patients were pooled. The PCR reaction was performed with a thermal cycler (iCycler; Bio Rad, Hercules, CA) in a total volume of 25  $\mu$ l. The PCR conditions were: denaturation at 95° C for 9 min; followed by 35 cycles at 95° C for 1 min; 58° C for 30 sec (Table 1); and 72° C for 1.5 min; a final extension step was then carried out at 72° C for 7 min. For heteroduplex formation, each PCR product (25  $\mu$ l) was denatured at 95° C for 5 min and gradually cooled to 25° C.

For analyses of a few samples, each of seven exonic regions was amplified simultaneously by PCR in a 96-well plate (96-well Multiplate, MLP-9601; MJ Research, Waltham, MA). Seven wells were used for each patient. Primer sets were designed to be effective using a single annealing temperature of 58° C (Table 11).

**Table 11. Primer sequences, product size, and PCR annealing and DHPLC analysis temperatures**

Exon		Primer sequences (5' to 3')	Product size (bp)	PCR T <sub>m</sub> (°C)	DHPLC T <sub>m</sub> (°C)	
1A	F	AGC ACA GGA GAG CTT TCC AGA GGA	302	58.0	61.9	(SEQ ID NO:65)
	R	CTC CAG GTC TAA GCG TTG G				(SEQ ID NO:66)
1B	F	CAG GCG ATG TCA GTC ATC CA	298	58.0	61.2, 64.5	(SEQ ID NO:67)
	R	TCT CAT TTT CTT GCC TTA GTC				(SEQ ID NO:68)
1C	F	GAA ACC OAA ACC AGA GAG	255	58.0	61.0, 63.5	(SEQ ID NO:69)
	R	ATA TCA COT GGT GAA CTC AGA GTC				(SEQ ID NO:70)
2A	F	CCT CAA CAT AGT CAA TCC TTG GGC	245	58.0	56.3, 59.3	(SEQ ID NO:71)
	R	ACA TGA ATA AAG ACC ATG TGG GCA				(SEQ ID NO:72)
3A	F	GAT TAT GGA TTA AGT GGT GCT TCG	375	58.0	59.3, 61.3, 62.3	(SEQ ID NO:73)
	R	TGT CTC GGT ATT CAG CTC AT				(SEQ ID NO:74)
3B	F	CAT ACT GCC TAG GCC ACT GGA	337	58.0	60.9, 61.4	(SEQ ID NO:75)
	R	ATT GGC GAC TGA CTG CTT AC				(SEQ ID NO:76)
3C	F	GAA TCT GGA ACT CGA ACA AA	333	58.0	59.7, 61.7	(SEQ ID NO:77)
	R	CTG AGC ATC TCC TTG TGG CAT				(SEQ ID NO:78)

#### Denaturing HPLC Analysis

5 For high-throughput analysis, a 25 µl volume of PCR products from the three patients was automatically injected into the chromatograph for analysis using the WAVE® System for DHPLC analysis (Transgenomic, Omaha, NE). The DHPLC melting temperatures are listed in Table 1. For analysis of

10 a small number of samples, following 96-well-plate PCR, the plate was next placed in a WAVE® System programmed to automatically analyze each well at two to three melting temperatures. Approximately 3 hrs was sufficient time to analyze one individual's sample.

15 When abnormal chromatographic patterns were detected in the pooled samples by the high-throughput protocol, the sample was reanalyzed individually in the WAVE® System. The

PCR product that showed the abnormal chromatographic pattern was then sequenced.

#### **Direct DNA Sequencing**

For direct sequencing, PCR products were purified  
5 with a QIA Quick PCR purification kit (Qiagen, Valencia, CA) to remove unused primers and precursors. The PCR products were directly sequenced with the same forward and reverse PCR amplification primers on an ABI310 automated sequencer using BigDye chemistry according to the  
10 manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

#### **Results**

##### **Screening of Pools of DNA in 171 Patients**

Four DHPLC tracing patterns in the Exon3C region  
15 were shown in Figure 2. The upper most pattern (A) has a normal appearance, while the middle pattern (B) showed a broad shoulder, and the lower patterns (C and D) had a characteristic double peak pattern indicative of sequence variations in this region. Sequencing analysis of samples B,  
20 C, and D revealed Thr448Pro, Pro481Ser, and Ala488Ala mutations (Table 12).

Four glaucoma-causing mutations were identified in 5 (2.9%) of 171 patients with POAG. In addition, eight polymorphisms and five synonymous codon changes were

identified (Table 12). One novel missense mutation, Phe369Leu detected in exon 3 (Figure 3) was not present in 100 normal Japanese subjects. The three other missense mutations, Ile360Asn, Ala363Thr, and Thr448Pro have been reported in Japanese patients with POAG.

Table 12. MYOC mutations and polymorphisms in patients with POAG and controls

	Exon	Sequence change	Amino acid change	Frequency	
				patients	controls
Mutations	3	c.1079T>A	Ile360Asn	1/171	0/100
	3	c.1087G>A	Ala363Thr	2/171	0/100
	3	c.1105T>C	Phe369Leu*	1/171	0/100
	3	c.1342A>C	Thr448Pro	1/171	0/100
Polymorphisms	1	c.34G>C	Gly12Arg	1/171	2/100
	1	c.57G>T	Gln19His	1/171	1/100
	1	c.136C>T	Arg46Stop	1/171	1/100
	1	c.210C>T	Val70Val <sup>†</sup>	2/171	0/100
	1	c.227G>A	Arg76Lys	14/171	9/100
	1	c.369C>T	Thr123Thr	1/171	0/100
	1	c.473G>A	Arg158Gln	1/171	1/100
	2	c.611C>T	Thr204Met	0/171	1/100
	2	c.624C>G	Asp208Glu	5/171	2/100
	3	c.864C>T	Ile288Ile	1/171	0/100
	3	c.1110G>A	Pro370Pro	0/171	1/100
	3	c.1441C>T	Pro481Ser	1/171	0/100
	3	c.1464C>T	Ala488Ala	3/171	1/100

\* Novel myocilin mutation; <sup>†</sup> novel myocilin polymorphism.

#### Screening of Individual Patients by Plate PCR followed by

#### 10 DHPLC

A DHPLC tracing from a patient with POAG is shown in Figure 4. In the exon3B region, an abnormal tracing indicative of sequence variation can be seen, which proved to represent a Phe369Leu mutation on direct sequencing.

Partial nucleotide sequences for MYOC exon 3 gene containing the targeted polymorphism is as follows:

**MYOC Exon 3, codon 369 (underlined) TTC(Phe) to CTC(Leu)**

301 actggaaagc acgggtgctg tgggtgactc ggggagcctc tatttcagg gcgctgagtc  
361 cagaactgtc ataagatatg agctgaatac cgagacagtg aaggctgaga aggaaatccc  
421 tggagctggc taccacggac agttcccgta ttcttggggt ggctacacgg acattgactt  
481 ggctgtggat gaagcaggcc tctgggtcat ttacagcacc gatgaggcca aagggtgccat  
541 tgtcctctcc aaactgaacc cagagaatct ggaactcgaa caaacctggg agacaacat  
(SEQ ID NO:79)

The nucleotide sequences of MYOC exon 1-3 are available from GenBank, Accession Nos. AB006686-AB006688

**Example 6 Variants in Optineurin Gene and their Association with Tumor Necrosis Factor- $\alpha$  Polymorphisms in Japanese Patients with Glaucoma**

**Purpose:** To investigate sequence variations in the optineurin (OPTN) gene and their association with TNF- $\alpha$  polymorphism in Japanese patients with glaucoma.

## **SUBJECTS AND METHODS**

### **Patients and Control Subjects**

A total of 629 blood samples were collected at seven institutions in Japan. There were 194 POAG patients, 217 NTG patients, and 218 normal controls, and none of the

subjects was related to others in this study. The patients whose age at diagnosis was less than 35 years and patients with over -5.5 D of myopia were excluded. POAG patients with *MYOC* mutations were also excluded.

5     **DNA Extraction and PCR Conditions**

Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 13 exonic coding regions of the *OPTN* gene were amplified by polymerase chain reaction (PCR) using the primer sets  
10     listed in Table 13. A 20-base GC-clamp was attached to some of the forward primers to detect mutations in the higher melting temperature domain by DHPLC analysis (Narayanawami G et. al., Genet Test. 2001;5:9-16). In high-throughput analysis, samples from three patients were pooled. PCR was  
15     performed with a thermal cycler (iCycler, Bio-Rad; Hercules, CA) in a total volume of 20 µl containing; 45 ng of genomic DNA, 2 µl GeneAmp 10x PCR buffer II, 2 µl of GeneAmp dNTP mix with a 2.0 mM concentration of each dNTP, 2.4 µl of a 25 mM MgCl<sub>2</sub> solution; 4 pmol of each primer, and 0.1 U of  
20     AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR conditions were; denaturation at 95° C for 9 min, followed by 35 cycles at 95° C for 1 min, 55° to 60° C for 30 sec (Table 13), and 72° C for 1 min and 30 sec, and a final extension step at 72° C for 7 min.



**Table 13. Primer sequences, PCR product sizes, and PCR annealing and DHPLC analysis temperatures**

Exon		Primer Sequences (5' to 3')	PCR product size (bp)	PCR Tm (°C)	DHPLC Tm (°C)	
4	F	CCAGTGGGTTTGTGGGACTCC	317	60	61.7	(SEQ ID NO:80)
	R	AAAGGGATGGCATTTTCTTGCA				(SEQ ID NO:81)
5	F	GTCCACTTTCTGGTGTGTGACT	277	55	58.7	(SEQ ID NO:82)
	R	CAACATCACAATGGATCG				(SEQ ID NO:83)
6	F	AGCCTTAGTTGATCTGTTCAATCA	293	60	57.0, 62.5	(SEQ ID NO:84)
	R	GTTTCATCTTCCAGGGGAGGCT				(SEQ ID NO:85)
7	F	GC-clamp AATCCCTTGCATTCTGTTTT	188	55	59.4, 61.4, 62.4	(SEQ ID NO:86)
	R	GTGACAAGCACCCAGTGACGA				(SEQ ID NO:87)
8	F	GC-clamp GGTTACTCTCTTCTAGTCTTTGGA	320	57	54.6, 58.5	(SEQ ID NO:88)
	R	GGGTGAAGTGTATGGTATCTTAATT				(SEQ ID NO:89)
9	F	GC-clamp GCTATTTCTCTTAAAGCCAAGAGA	242	55	57.4, 59.4	(SEQ ID NO:90)
	R	CAGTGGCTGGAAGTACTCTGT				(SEQ ID NO:91)
10	F	GC-clamp GTCAGATGATAATTGTACAGATAT	227	55	57.8, 59.8	(SEQ ID NO:92)
	R	AATGTATATTTCAAAGGAGGATAAA				(SEQ ID NO:93)
11	F	CCACTCGCAGCTAAAGGAGCA	286	60	57.5, 59.5	(SEQ ID NO:94)
	R	CAAAATCCGAATTCGAATCTGTATAA				(SEQ ID NO:95)
12	F	GC-clamp GGTGGGAGGCAAGACTATAAGTT	233	60	55.5, 56.5	(SEQ ID NO:96)
	R	TTCTGTTCTTACTAGGCTATGGAA				(SEQ ID NO:97)
13	F	CAGGCAGAAATTATTTCAAACCAT	264	60	58.9, 61.9	(SEQ ID NO:98)
	R	CGAGAATACAATCAGGGCTGG				(SEQ ID NO:99)
14	F	GCACTACCTCTCATCGCATAAACA	260	60	56.7, 59.7	(SEQ ID NO:100)
	R	GGCCATGCTGATGTGAGTCT				(SEQ ID NO:101)
15	F	GC-clamp GGACTGTCTGCTCAGTGTGTCA	282	60	56.0, 59.0, 61.0	(SEQ ID NO:102)
	R	GGTGCCTTGATTTGGAATCCA				(SEQ ID NO:103)
16	F	GC-clamp CACAAGTCTGCAAAATGGAAC	294	60	61.7	(SEQ ID NO:104)
	R	GAGGCAAAATATTGAGTGAAACA				(SEQ ID NO:105)

GC-clamp: CGCCCGCCGCCGCCGCCG

## 5 Denaturing HPLC Analysis

DHPLC analysis was performed using the WAVE<sup>®</sup> SYSTEMS (Transgenomic, Omaha, NE). For heteroduplex formation, products of each PCR (20 µl) were denatured at 95° C for 5 min and gradually cooled to 25° C. The annealed PCR products from the three mixed samples were automatically injected into a DNASep<sup>®</sup> cartridge (Transgenomic, Omaha, NE).

Buffer A (Transgenomic, Omaha, NE) was made up of 0.1 M triethylammonium acetate (TEAA), and Buffer B of 0.1 M TEAA and 25% acetonitrile. Analysis was carried out at a flow rate of 0.9 ml/min and the Buffer B gradient increased by 2%/min for 4.5 min. Elution of DNA fragments from the cartridge was detected by absorbance at 260 nm. The temperatures used for the analysis were selected according to the sequences of the DNA fragments. The WAVEMAKER software (v.4.1, Transgenomic, Omaha, NE) predicted the melting behavior of the DNA fragments at various temperatures. The predicted melting domains within the DNA fragment determined the temperatures for the DHPLC analysis (Table 13). When abnormal chromatographic patterns were detected in a pool of three samples, each of the three samples was re-analyzed individually in the WAVE<sup>®</sup> SYSTEM. Then, the PCR product that showed an abnormal chromatographic pattern was sequenced. Once a correlation between abnormal chromatographic patterns and base changes was confirmed by direct sequencing analysis, additional sequencing analyses were not performed when any of the known abnormal chromatographic patterns were observed in the DHPLC analysis.

#### **Direct DNA Sequencing**

To detect mutations by direct sequencing, the PCR

products were first purified with the QIAquick PCR Purification Kit (QIAGEN, Valenica, CA, USA) to remove unreacted primers and precursors. The sequencing reactions were then performed using the ABI PRISM BigDye Terminator (v.3.1) Cycle Sequencing Kit, according to the manufacturer's protocol (Applied Biosystems). The data were collected by the ABI PRISM 310 Genetic Analyzer and analyzed by the ABI PRISM sequencing analysis program (v.3.7).

10 **Genotyping OPTN c.412G>A (Thr34Thr) Polymorphism**

The G to A substitution at position c.412 in exon 4 of the OPTN gene was detected by using restriction enzyme, *HpyCH<sub>4</sub>IV* (New England BioLabs, Beverly, MA), with the same primers listed in Table 13 for the DHPLC analysis. The G allele sequence was cut into two fragments (188 bp + 129 bp) by *HpyCH<sub>4</sub>IV*, while the A allele sequence remained intact (317 bp). The polymorphism was confirmed by restriction-enzyme assay and the chromatographic pattern of DHPLC.

20 **Genotyping OPTN c.603T>A (Met98Lys) Polymorphism**

The T to A substitution at position c.603 in exon 5 of the OPTN gene was detected by restriction enzyme, *Stu I* (TaKaRa, Shiga, Japan), using the same primers as for the DHPLC analysis (Table 13). The A allele sequence was cut

into two fragments (175 bp + 102 bp) by *Stu* I, while the T allele sequence remained intact (277 bp). The polymorphism was confirmed by restriction-enzyme assay and the chromatographic pattern of DHPLC.

5     **Genotyping OPTN c.1944G>A (Arg545Gln) Polymorphism**

The G to A substitution at position c.1944 in exon 16 of the *OPTN* gene was analyzed by the Invader assay provided by the Research Department of R&D Center, BML (Saitama, Japan). The polymorphism was confirmed by  
10    Invader® assay and by the chromatographic pattern of DHPLC.  
**Genotyping TNF- $\alpha$  -308G>A Polymorphism**

Genotyping the -308G>A polymorphism in the TNF- $\alpha$  promoter region was performed by using restriction enzyme *Nco*I (New England BioLabs, Beverly, MA), with the forward  
15    primer, 5'-AGGCAATAGGTTTGGAGGGCCAT-3' (SEQ ID NO:106), and the reverse primer, 5'-GTAGTGGGCCCTGCACCTTCT -3' (SEQ ID NO:107). The forward primer contained one nucleotide mismatch (bold and underlined), which allowed the use of the restriction enzyme. The G allele sequence was cut into  
20    two fragments (192 bp +20 bp) by *Nco*I while the A allele sequence remained intact (212 bp).

**Genotyping TNF- $\alpha$  -857C>T Polymorphism**

Genotyping the -857C>T polymorphism in the TNF- $\alpha$  promoter region was performed by using restriction enzyme

*HincII* (TaKaRa, Shiga, Japan), with the forward primer, 5'-AAGTCGAGTATGGGGACCCCCGTTAA-3' (SEQ ID NO:108), and the reverse primer, 5'-CCCCAGTGTGTGGCCATATCTTCTT-3' (SEQ ID NO:109). The forward primer contained one nucleotide mismatch (bold and underlined), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (106 bp +25 bp) by *HincII*, while the T allele sequence remained intact (131 bp). Transcriptional activity of the -857T allele was significantly greater than that of -857C allele.

#### Genotyping TNF- $\alpha$ -863C>A Polymorphism

Genotyping the -863C>A polymorphism in the TNF- $\alpha$  promoter region was done by using restriction enzyme *EcoNI* (New England BioLabs, Beverly, MA) with the forward primer, 5'-GCTGAGAAGATGAAGGAAAAGTC-3' (SEQ ID NO:110), and the reverse primer, 5'-CCTCTACATGGCCCTGTCCT-3' (SEQ ID NO:111). The reverse primer contained one nucleotide mismatch (bold and underlined), which allowed the use of the restriction enzyme. The C allele sequence was cut into two fragments (183 bp +23 bp) by *EcoNI*, while the A allele sequence remained intact (206 bp). Transcriptional activity of the -863A allele was significantly greater than that of -863C allele.

#### Statistical Analyses

The frequencies of the genotypes and alleles in patients and controls were compared with the chi-square test and Fisher's exact test. The odds ratio and 95% confidence intervals (CI) also were calculated. The Hardy-  
5 Weinberg equilibrium for the observed frequencies was also calculated. Comparisons of the clinical characteristics between the two groups were performed using Mann-Whitney *U* test or Student's unpaired *t*-test when appropriate. Logarithmic transformation was performed on skewed  
10 distribution clinical data which were the IOP at diagnosis of POAG, visual field score at diagnosis of NTG, and POAG to obtain a normal distribution for performing analysis of variance (ANOVA). One-way ANOVA was used to compare three clinical characteristics among patients with 4 different  
15 combinations of the  $TNF-\alpha/-857C>T$  and optineurin/412G>A genotypes, or the  $TNF-\alpha/-863C>A$  and optineurin/603T>A genotypes (see Table 17).

Statistical analysis was performed with SPSS program (SPSS Inc., Chicago, USA ). A *P* value of  $<0.05$  was  
20 considered to be significant.

## RESULTS

### OPTN Variants in Japanese Subjects

A total of 629 Japanese subjects were studied, and the results are presented in Table 14.

Table 14. OPTN variants observed in glaucoma patients and control subjects

Location	Sequence Changes	Codon Changes	Frequency in Subjects (%)		
			POAG	NTG	Control
Exon 4	c.386C>G	His26Asp	1 / 201 (0.5)	0 / 232 (0)	0 / 218 (0)
Exon 4	c.449-451delCTC	Leu47del	0 / 201 (0)	0 / 232 (0)	1 / 218 (0.5)
Exon 5	c.603T>A	Met98Lys	33 / 201 (16.4)	50 / 232 (21.6)	36 / 218 (16.5)
Exon 16	c.1944G>A	Arg545Gln	14 / 192 (7.3)	15 / 222 (6.8)	11 / 214 (5.1)
Exon 4	c.412G>A	Thr34Thr	69 / 201 (34.3)	74 / 232 (31.9)	52 / 218 (23.9)
Exon 4	c.421G>A	Pro37Pro	0 / 201 (0)	1 / 232 (0.4)	0 / 218 (0)
Exon 4	c.457C>T	Thr49Thr	2 / 201 (1)	0 / 232 (0)	0 / 218 (0)
Exon 16	c.2023C>T	His571His	0 / 162 (0)	0 / 193 (0)	2 / 196 (1.0)
Intron 4	c.476+15C>A		0 / 201 (0)	0 / 232 (0)	1 / 218 (0.5)
Intron 6	c.863-10G>A *		N/C†	N/C	N/C
Intron 6	c.863-5C>T *		N/C	N/C	N/C
Intron 8	c.1089+20G>A		4 / 133 (3.0)	11 / 172 (6.4)	4 / 126 (3.2)
Intron 9	c.1192+19C>T		0 / 133 (0)	4 / 172 (2.3)	3 / 130 (2.3)
Intron 11	c.1458+28G>C		1 / 133 (0.8)	4 / 172 (2.3)	0 / 157 (0)
Intron 15	c.1922+10G>A		2 / 133 (1.5)	4 / 172 (2.3)	1 / 157 (0.6)
Intron 15	c.1922+12G>C		0 / 133 (0)	1 / 172 (0.6)	0 / 157 (0)
Intron 15	c.1923-48C>A *		N/C	N/C	N/C

\* Sequence variation was found by direct sequencing analysis.

† Not checked

5               Seventeen sequence changes were identified in the  
glaucoma patients and control subjects. Among these, three  
were missense changes, one was a deletion of one amino acid  
residue, four were synonymous codon changes, and nine were  
changes in noncoding sequences. One possible disease  
causing-mutation, His26Asp, was identified in one POAG  
10 proband and was not present in the 218 normal Japanese  
controls. Her brother aged 55 harbored the mutation and was  
diagnosed as NTG. Her brother's daughter aged 23 also had

the mutation and showed cupping of the optic nerve head with a cup/disk ratio of 0.7 with no sign of visual field defect by Humphrey perimetry .

A deletion of Leu47 (3-bp deletion, CTC) was found  
5 in 1 control. A Met98Lys was identified in 33 POAG patients,  
48 NTG patients, and 36 controls, and an Arg545Gln was  
identified in 11 POAG patients, 15 NTG patients, and 11  
controls.

Four synonymous nucleotide substitutions, c.412G>A  
10 (Thr34Thr), c.421G>A (Pro37Pro), c.457C>T (Thr49Thr), and  
c.2023C>T (His571His) were found. The Thr34Thr substitution  
was present in 69 (35.6%) POAG patients, 69 (31.8%) NTG  
patients, and 52 (23.9%) controls, and the Pro37Pro was  
found in 1 NTG patient. The Thr49Thr was identified in 1  
15 POAG patient, and the His571His was present in 2 controls.

#### Distribution of OPTN Variants in Japanese Subjects

The Thr34Thr (c.412G>A) polymorphism was  
significantly associated with POAG and NTG (Table 15). A  
significant association was found in patients with POAG ( $P$   
20 = 0.009 in genotype frequency: G/G vs G/A+A/A, and  $P$  = 0.003  
in allele frequency). No significant difference was  
detected between glaucoma patients and controls in either  
genotype or allele frequency for the Met98Lys (c.603T>A) or  
the Arg545Gln (c.1944G>A) polymorphisms. However, the



Met98Lys polymorphism had a higher tendency to be associated with NTG than with POAG. The observed genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium.

Table 15. Genotype distribution and allele frequency of optineurin gene polymorphisms in glaucoma patients and controls c. 412G>A (Thr34Thr)

Phenotype	n	Genotype frequency (%)			Genotype frequency (%)			Allele frequency (%)		
		GG	GA	AA	P value*	GG	GA+AA	AA	P value*	A
POAG	194	125 (64.4)	61 (31.4)	8 (4.1)	0.011 ‡	125 (64.4)	89 (35.8)	0.008 §	8 (4.1)	0.051
										311 (80.2)
										77 (19.8)
										0.003 §
NTG	217	148 (68.2)	62 (28.6)	7 (3.2)	0.078	148 (68.2)	69 (31.8)	0.064	7 (3.2)	0.105
										358 (82.5)
										76 (17.5)
										0.034 ‡
Control	218	166 (76.1)	50 (22.9)	2 (1.0)		166 (76.1)	62 (23.9)		2 (1.0)	382 (91.6)
										64 (12.4)

  

c.603T>A (Met98Lys)										
Phenotype	n	Genotype frequency (%)			P value*	Genotype frequency (%)			P value*	Allele frequency (%)
		TT	T/A	AA		TT	T/A+AA	AA		
POAG	194	161 (83.0)	32 (16.5)	1 (0.5)	0.990	161 (83.0)	33 (17.0)	0.893	1 (0.5)	354 (81.2)
										34 (8.8)
										0.888
NTG	217	165 (77.9)	43 (19.8)	5 (2.3)	0.133	165 (77.9)	48 (22.1)	0.139	0.122	331 (87.6)
										53 (12.2)
										0.071
Control	218	182 (83.5)	35 (16.0)	1 (0.5)		182 (83.5)	36 (16.5)		1 (0.5)	368 (81.5)
										37 (8.5)

\* P value for  $\chi^2$  test.  
 † P value for Fisher's exact test.  
 ‡  $p < 0.05$   
 §  $p < 0.01$

Three clinical characteristics of the glaucoma patients, viz., age at diagnosis, IOP at diagnosis, and visual field score at diagnosis, were examined for association with c.412G>A (Thr34Thr) or c.603T>A (Met98Lys) polymorphisms (Table 16). The glaucoma patients did not show an association with the clinical characteristics with the c.412G>A polymorphism. POAG patients with the G/A+A/A genotype (or 412A carriers) tended to have more advanced visual field scores than those with the G/G genotype (or non-412A carriers;  $P = 0.093$ ). POAG patients with the 603T>A polymorphism showed a weak association with age at diagnosis ( $P = 0.046$ ).

Table16 Comparison of clinical characteristics of glaucoma patients according to *OPTN* genotypes

**c.412G>A (Thr34Thr)**

	Phenotype Variable	G/G	G/A+A/A	<i>P</i> value*
POAG	Age at diagnosis (ys)	58.1 ± 11.8 (n = 123)	58.8 ± 12.6 (n = 69)	0.863
	IOP at diagnosis (mm Hg)	27.0 ± 6.5 (n = 112)	26.1 ± 5.0 (n = 80)	0.380
	Visual field score at diagnosis	3.0 ± 0.9 (n = 125)	3.2 ± 0.9 (n = 69)	0.093
NTG	Age at diagnosis (ys)	58.7 ± 11.7 (n = 148)	56.6 ± 11.2 (n = 69)	0.206
	IOP at diagnosis (mm Hg)	16.4 ± 2.6 (n = 139)	16.6 ± 2.2 (n = 67)	0.848
	Visual field score at diagnosis	2.8 ± 0.7 (n = 148)	2.7 ± 0.7 (n = 69)	0.135

**c.603T>A (Met98Lys)**

	Phenotype Variable	T/T	T/A+A/A	<i>P</i> value*
POAG	Age at diagnosis (ys)	57.6 ± 11.9 (n = 159)	62.2 ± 12.4 (n = 33)	0.046†
	IOP at diagnosis (mm Hg)	26.8 ± 5.8 (n = 143)	26.5 ± 7.1 (n = 29)	0.931
	Visual field score at diagnosis	3.1 ± 0.9 (n = 161)	3.2 ± 0.9 (n = 33)	0.280
NTG	Age at diagnosis (ys)	58.4 ± 11.6 (n = 169)	56.6 ± 11.6 (n = 48)	0.304
	IOP at diagnosis (mm Hg)	16.4 ± 2.4 (n = 160)	16.8 ± 2.6 (n = 46)	0.270
	Visual field score at diagnosis	2.8 ± 0.7 (n = 169)	2.8 ± 0.6 (n = 48)	0.318

\* *P* values for Mann-Whitney U test.

†  $P < 0.05$

**Association between OPTN Polymorphism and TNF- $\alpha$   
Polymorphism in Glaucoma Patients**

No significant difference in genotype or allele  
5 frequency was noted between patients and controls for the  
three polymorphisms of the -308G>A, -857C>T or -863C>A. In  
addition, the glaucoma patients did not show an association  
with the clinical characteristics for the three  
polymorphisms (data not shown). The observed genotype  
10 frequencies were in agreement with those predicted by the  
Hardy-Weinberg equilibrium.

However, among individuals with the C/T+T/T genotype  
(or -857T carriers) in the TNF- $\alpha$  gene, 44.1 % of POAG  
patients were G/A+A/A genotypes (or 412A carriers) in the  
15 OPTN gene compared to 21.6 % of controls (Table 17). This  
difference in frequency was significant ( $P = 0.006$ ). Among  
individuals with the C/A+A/A genotype (or -863A carriers)  
in the TNF- $\alpha$  gene, 603A carriers (or Lys98 carriers) in the  
OPTN gene were significantly associated with POAG as well  
20 as NTG ( $P = 0.008$  and  $0.027$ , respectively).

Table 17 Distribution of optineurin genotypes (c.412G>A and c.603T>A) according to TNF- $\alpha$  genotypes (-857C>T and -863C>A)

## c.412G&gt;A (Thr34Thr)

Phenotype	-857C>T		C/C (%)		P value*	Odds ratio 95 % CI	C/T+T/T (%)		P value*	Odds ratio 95 % CI
	c.412G>A	G/G	G/A + A/A				G/G	G/A + A/A		
POAG	92 (68.1)	43 (31.9)	0.204	1.40 (0.83-2.37)	33 (55.9)	26 (44.1)	0.006‡	2.86 (1.34-6.08)		
NTG	97 (65.5)	51 (34.5)	0.077	1.58 (0.95-2.62)	51 (73.9)	18 (26.1)	0.531	1.28 (0.59-2.77)		
Control	108 (75.0)	36 (25.0)			58 (78.4)	16 (21.6)				

Phenotype	-863C>A		C/C (%)		P value*	Odds ratio 95 % CI	C/A+A/A(%)		P value*	Odds ratio 95 % CI
	c.412G>A	G/G	G/G + A/A				G/G	G/A + A/A		
POAG	91 (64.5)	50 (35.5)	0.017	1.84 (1.11-3.05)	34 (64.2)	19 (35.8)	0.280	1.56 (0.89-3.53)		
NTG	110 (69.2)	49 (30.8)	0.114	1.49 (0.91-2.46)	38 (65.5)	20 (34.5)	0.341	1.47 (0.66-3.28)		
Control	124 (77.0)	37 (23.0)			42 (73.7)	15 (26.3)				

## c.603T&gt;A (Met98Lys)

Phenotype	-857C>T		C/C (%)		P value*	Odds ratio 95 % CI	C/T+T/T (%)		P value*	Odds ratio 95 % CI
	c.603T>A	T/T	T/A + A/A				T/T	T/A + A/A		
POAG	112 (83.0)	23 (17.0)	0.811	1.08 (0.57-2.03)	49 (83.1)	10 (16.9)	0.925	0.95 (0.39-2.37)		
NTG	111 (75.0)	37 (25.0)	0.056	1.75 (0.98-3.13)	58 (84.1)	11 (15.9)	0.795	0.89 (0.37-2.14)		
Control	121 (84.0)	23 (16.0)			61 (82.4)	13 (17.6)				

Phenotype	-863C>A		C/C (%)		Odds ratio 95 % CI	C/A+A/A (%)		Odds ratio 95 % CI
	c.603T>A	T/T	T/A + A/A	P value*		T/T	T/A + A/A	
POAG	123 (87.2)	18 (12.8)	0.127	0.61 (0.33-1.15)	38 (71.7)	15 (28.3)	0.008‡	4.11 (1.37-12.27)
NTG	125 (78.6)	34 (21.4)	0.636	1.14 (0.66-1.97)	44 (75.8)	14 (24.1)	0.027†	3.31 (1.10-9.91)
Control	130 (80.7)	31 (19.3)			52 (81.2)	5 (8.8)		

\* P values for  $\chi^2$  test.

† P &lt; 0.05

‡ P &lt; 0.01

The clinical characteristics of these combined genotypes, such as age at diagnosis, IOP at diagnosis, and visual field score at diagnosis are shown in Table 18. The POAG patients who were TNF- $\alpha$ -857T and optineurin/412A carriers had significantly worse ( $P = 0.020$ ) visual field scores than those who were TNF- $\alpha$ -857T and non-optineurin/412A carriers. However, there was no significant difference in the three clinical features of POAG patients

among the four genotypes of combined -857T>A and c.412G>A polymorphisms (Table 6) by one-way ANOVA:  $P = 0.823$  for age at diagnosis;  $P = 0.692$  for IOP at diagnosis; and  $P = 0.152$  for visual field score at diagnosis.

5 POAG patients who were TNF- $\alpha$ /-863A and optineurin/603A carriers had significantly worse ( $P = 0.026$ ) visual field scores than those who were TNF- $\alpha$ /-863A and non-optineurin/603A carriers. However, there was no significant difference in the visual field score of POAG  
10 patients among the four genotypes of combined -863C>A and -603T>A polymorphisms (Table 6, one-way ANOVA:  $P = 0.200$ ).

Table 18 Comparison of clinical characteristics of glaucoma patients according to TNF- $\alpha$  genotypes (-857T and -863A) and optineurin genotypes (412A and 603A)

c.412G>A (Thr34Thr)				
(TNF- $\alpha$ genotypes)		C/T+T/T (-857T carrier)		P value*
(OPTN genotypes)		G/G	G/A+A/A	
POAG	Age at diagnosis (ys)	57.1 $\pm$ 10.7 (n = 32)	57.6 $\pm$ 13.1 (n = 26)	0.802
	IOP at diagnosis (mm Hg)	26.4 $\pm$ 6.1 (n = 30)	26.4 $\pm$ 5.5 (n = 20)	0.786
	Visual field score	2.9 $\pm$ 0.9 (n = 33)	3.3 $\pm$ 0.8 (n = 26)	0.020†
NTG	Age at diagnosis (ys)	58.4 $\pm$ 11.1 (n = 51)	59.3 $\pm$ 10.5 (n = 18)	0.790
	IOP at diagnosis (mm Hg)	16.4 $\pm$ 2.6 (n = 46)	16.1 $\pm$ 2.3 (n = 17)	0.520
	Visual field score	2.8 $\pm$ 0.8 (n = 51)	2.6 $\pm$ 0.5 (n = 18)	0.335
c.603T>A (Met98Lys)				
(TNF- $\alpha$ genotypes)		C/A+A/A (-863A carrier)		P value*
(OPTN genotypes)		T/T	T/A+A/A	
POAG	Age at diagnosis (ys)	56.3 $\pm$ 10.5 (n = 38)	62.0 $\pm$ 13.8 (n = 15)	0.074
	IOP at diagnosis (mm Hg)	27.9 $\pm$ 6.5 (n = 36)	26.9 $\pm$ 8.7 (n = 14)	0.488
	Visual field score	3.0 $\pm$ 0.8 (n = 38)	3.5 $\pm$ 0.9 (n = 15)	0.026†
NTG	Age at diagnosis (ys)	57.9 $\pm$ 11.4 (n = 44)	56.9 $\pm$ 11.9 (n = 14)	0.579
	IOP at diagnosis (mm Hg)	16.2 $\pm$ 2.4 (n = 40)	16.9 $\pm$ 2.4 (n = 14)	0.364
	Visual field score	2.9 $\pm$ 0.5 (n = 44)	2.7 $\pm$ 0.6 (n = 14)	0.296

\* P values for Mann-Whitney U test.

†  $P < 0.05$

Partial nucleotide sequence of OPTN exon 4, comprising  
the targeted polymorphism, 412G>A (underlined)

caacagtgc tttccacag gaacttctgc aatgtcccat caacctctca gctgcctcac  
tgaaaaggag gacagcccca gtgaaagcac aggaaatgga cccccccacc tggcccaccc  
5 aaacctggac acgtttaccc cggaggagct gctgcagcag atgaaagagc tcttgaccga  
gaaccaccag ctgaaagggtg agcagggtctg gccctctgtg gcccattca tcttgggcct  
(SEQ ID NO:112)

Sequence of OPTN gene, GeneBank Accession No.

AF423071

10 1 atcccggtcg ggagttctct ccaggcggca cgatgccgag gaaacagtga ccttgagcga  
61 agccaagccg ggcggcaggt gtggctttga tagctggtgg tgccacttcc tggccttgga  
121 tgagccgtac gctctgttaa acccaacttc ctcacctttg aaacagctgc ctggctcagc  
181 attaatgaag attagtcatg gacaggcctg gtgtgctgag tccgcacata gaagaatcaa  
241 aaatgtccaa aatgtaactg gagagaaagt gggcaacttt tggagtgact tttccacagg  
15 301 aacttctgca atgtcccatc aacctctcag ctgcctcact gaaaaggagg acagcccag  
361 tgaaagcaca ggaatggac cccccacct ggcccacca aactggaca cgtttacccc  
421 ggaggagctg ctgcagcaga tgaagagct cctgaccgag aaccaccagc tgaaagaagc  
481 catgaagcta aataatcaag ccatgaaagg gagatttgag gagctttcgg cctggacaga  
541 gaaacagaag gaagaacgcc agttttttga gatacagagc aaagaagcaa aagagcgtct  
20 601 aatggccttg agtcatgaga atgagaaatt gaaggaagag ctttgaaaaa taaaagggaa  
661 atcagaaagg tcatctgagg accccactga tgactccagg ctteccaggg ccgaagcggg  
721 gcaggaaaag gaccagctca ggaccaggt ggtgaggcta caagcagaga aggcagacct  
781 gttgggcctc gtgtctgaac tgcagctcaa gctgaactcc agcggtctct cagaagattc  
841 ctttgttgaa attaggatgg ctgaaggaga agcagaaggg tcagtataag aaatcaagca  
25 901 tagtcctggg cccacagaaa cagtctccac tggcacggca ttgtctaaat ataggagcag

961 atctgcagat gggccaaga attacttcga acatgaggag ttaactgtga gccagctcct  
 1021 gctgtgccta aggggaaggga atcagaaggt ggagagactt gaagttgcac tcaaggaggc  
 1081 caaagaaaga gtttcagatt ttgaaaagaa aacaagtaat cgttctgaga ttgaaaccca  
 1141 gacagagggg agcacagaga aagagaatga tgaagagaaa ggcccgagaga ctgttggaag  
 5 1201 cgaagtggaa gcactgaacc tccagggtgac atctctgttt aaggagcttc aagaggctca  
 1261 tacaaaaactc agcgaagctg agctaataa gaagagactt caagaaaagt gtcaggccct  
 1321 tgaaaggaaa aattctgcaa ttccatcaga gttgaatgaa aagcaagagc ttgtttatac  
 1381 taacaaaaag ttagagctac aagtggaaag catgctatca gaaatcaaaa tggaacaggc  
 1441 taaaacagag gatgaaaagt ccaaataac tgtgctacag atgacacaca acaagcttct  
 10 1501 tcaagaacat aataatgcat tgaaaaaat tgaggaaacta acaagaaaag agtcagaaaa  
 1561 agtggacagg gcagtgtgta aggaactgag tgaaaaactg gaactggcag agaaggctct  
 1621 ggcttccaaa cagctgcaaa tggatgaaat gaagcaaacc attgccaaagc aggaagagga  
 1681 cctggaaacc atgaccatcc tcagggtcca gatggaagtt tactgttctg attttcatgc  
 1741 tgaaagagca gcgagagaga aaattcatga ggaaaaggag caactggcat tgcagctggc  
 15 1801 agttctgctg aaagagaatg atgctttcga agacggaggc aggcagtcct tgatggagat  
 1861 gcagagtcgt catggggcga gaacaagtga ctctgaccag caggcttacc ttgttcaaa  
 1921 aggagctgag gacagggact ggcggcaaca gcggaatatt ccgattcatt cctgecccaa  
 1981 gtgtggagag gttctgctg acatagacac gttacagatt cagctgatgg attgcatcat  
 2041 ttaagtgttg atgtatcacc tccccaaaac tgttggt (SEQ ID NO:113)

20 Partial nucleotide sequence for TNF- $\alpha$  gene comprising  
 the targeted polymorphic position is as follows:

TNF- $\alpha$  -863C>A; -857C>T (underlined)

3121 ccacatgtag cggctctgag gaatgggtta caggagacct ctggggagat gtgaccacag  
 3181 caatgggtag gagaatgtcc agggctatga aagtcagta tggggacccc ccettaagga

3241 agacagggcc atgtagaggg cccaggggag tgaaagagcc tccaggacct ccaggatagg  
3301 aatacagggg acgtttaaga agatatggcc acacactggg gccttgagaa gtgagagctt  
(SEQ ID NO:114)

5     **Example 7. Effect of Oral Angiotensin II Receptor Blocker  
on IOP in Normal Subjects and Its Association with SNPs in  
AT1R and AT2R Genes**

**Example 7-1.**

**Methods**

10           Relationship between polymorphism at nucleotide number  
3123 (C or A) of the angiotensin II receptor 2 gene  
(AT2R) on chromosome-X and the effect of candesartan  
cilexetil, an angiotensin II receptor blocker was examined.  
This study was performed on 20 healthy volunteers (13 men  
15   and 8 women) without systemic and eye diseases. Among them,  
9 men had C, 4 men had A, 4 women had CC and 4 women had CA  
genotype at the polymorphic point. The each subject was  
given candesartan cilexetil orally and the IOP was recorded  
from 1 to 24 hours after the administration.

20     **RESULTS**

Change in Intraocular pressure 1-24 hours after the  
drug administration is shown in Table 19.



Table 19.

time 0	Lowering IOP mmHg								AT2R 3123C/A			
Base Line	1 Hr	2 Hr	3 Hr	4 Hr	5 Hr	6 Hr	24 Hr	M	A	F	F	
0	-2	-1	-3	-2	-1	-1	-1		A			I
0	-2	-2	0	0	-1	1	1		A			
0	1	1	0	0	-2	-2	0		A			
0	0	0	-2	1	0	0	-1	C				
0	-1	-3	-5	-2	-3	-3	-3	C			CA	II
0	0	-3	-2	-4	-3	0	0					
0	-1	-1	-4	-3	-4	-3	1	C				
0	-2	-4	-4	-4	-4	-5	-2	C				
0	-2	-3	-3	-2	-2	1	2			CC		III
0	-2	-3	-2	-5	-3	-3	0	C			CA	
0	-4	-6	-6	-6	-6	-4	-5				CA	
0	-4	-6	-6	-8	-5	-5	-4				CA	
0	-2	-3	-6	-5	-6	-3	-3	C				III
0	-2	-4	-4	-6	-3	-4	-5				CA	
0	-4	-6	-6	-7	-6	-6	-2			CC		
0	-4	-6	-5	-3	-5	-4	-3	C				
0	-1	-4	-5	-3	-6	-4	0			CC		III
0	-2	-4	-7	-6	-7	-6	-3			CC		
0	-2	-7	-8	-4	-6	-6	-1	C				
0	-8	-8	-8	-12	-12	-12	-12		A			

  

	IOP Lowering Effect	genotype
Group I	-	3 of 4 had A
Group II	+	5 of 6 had C or CC
Group III	++	7 of 11 had C or CC

5 In male, oral administration of candesartan cilexetil hardly lowered the IOP of 75% of those with A genotype at nucleotide 3123 of AT2R gene, whereas the IOP of 100% of those with C genotype was effectively lowered. In female, oral administration of candesartan cilexetil was

10 effectively lower the IOP of 100% of those with CC genotype.

This result suggest that nucleotide 3123 of AT2 (AGTR2) gene polymorphism associate with the effect of candesartan cilexetil.

Example 7-2.

#### 15 Methods

This study was performed on 20 healthy volunteers (13

men and 7 woman, age 23 to 28 years) without systemic and eye diseases. In the morning (10:00 hr), each subject was given either 12 mg oral candesartan cilexetil (Blopresse®, Takeda, Japan) or the placebo in a randomized crossover  
5 double-blind fashion.

The baseline heart rate, systolic/diastolic arterial pressures (SBP/DBP), and IOP were recorded. The subjects then received oral candesartan cilexetil or placebo, and measurements were repeated hourly for 6 hr and after 24 hr.  
10 One month later, each subject received the alternative treatment. Only the right eye was measured and analyzed.

The ocular perfusion pressure (OPP) is defined as the difference between the pressure in the arteries entering the tissue and the veins leaving it. The OPP can  
15 be approximated by the following formula using the mean blood pressure (BPM) and the IOP.

$$\text{OPP} = 2/3 \times \text{BPM} - \text{IOP}, \text{ where } \text{BPM} = \text{DBP} + 1/3 \times (\text{SBP} - \text{DBP}).$$

A search for polymorphisms in ATR1 and ATR2 was  
20 performed in the 20 subjects and correlated with the changes in the IOP. This research followed the tenets of the Declaration of Helsinki. Written informed consent was obtained after the nature and possible consequences of the study were explained. Where applicable, the research was

approved by the institutional human experimentation committee for analysis of DNA.

### ***Statistical Analysis***

Statistical analysis of the results following ARB  
5 was performed with StatView (SAS Institute, USA) using  
repeated measure ANOVA test. ANOVA test with Bonferroni  
correction was used for statistical analysis of each IOP  
values: a P value  $<0.0004$  was considered to be  
statistically significant.

### 10 **RESULTS**

The changes in the IOP after oral candesartan  
cilexetil or placebo are shown in Figure 5A. The IOP in the  
subjects who received the placebo was not altered  
significantly. On the other hand, as early as 1 hr after  
15 oral candesartan cilexetil, the IOP had fallen  
significantly and remained low for 5 hr ( $P < 0.0001$ )  
compared with placebo. Candesartan cilexetil did not  
significantly affect perfusion pressures (Fig. 5B). No  
significant change in SBP, DBP, and heart rate was detected  
20 after a single oral dose of candesartan cilexetil or  
placebo (data not shown).

The changes in the IOP after oral candesartan  
cilexetil in each of the 20 subjects are shown in Figure 5C.  
There was no significant association between the effects of

candesartan cilexetil and the three SNPs in the ATR1 gene in the 20 control subjects (Table 19-2). For the ATR2 genotype, however, 4 men with the A genotype showed a reduction of the IOP by  $2.3 \pm 0.5$  mmHg, which was the same value as that of subjects who received placebo, and a significantly less decrease in the IOP than in the 9 men with the C genotype ( $5.0 \pm 1.1$  mmHg,  $P = 0.014$ ). No woman had the AA genotype in this study.

Table 19-2. Effects of angiotensin II receptor blocker on intraocular pressure in association with genotypes of the angiotensin II receptor genes

Polymorphisms	Genotype	Number (eyes)	Maximum reduction of IOP (mmHg)	P*
AGTR1 -713T>G	TT	18	$4.9 \pm 1.8$	$P=0.898$
	TG	2	$5.0 \pm 4.2$	
	GG	0	0	
AGTR1 -521C>T	CC	18	$4.9 \pm 1.8$	$P^{\dagger}=0.117$
	CT	1	2	
	TT	1	8	
AGTR1 1166A>C	AA	18	$5.1 \pm 2.0$	$P=0.405$
	AC	2	$5.2 \pm 1.6$	
	CC	0	0	
AGTR2 3123C>A	C (male)	9	$5.0 \pm 1.1$	$P=0.014 \ddagger$
	A (male)	4	$2.3 \pm 0.5$	
	CC (female)	3	$7.0 \pm 1.0$	$P=0.354$
	CA (female)	4	$6.0 \pm 1.6$	
	AA (female)	0	0	

\* P value for Mann-Whitney U test

† P value for Kruskal-Wallis test

‡  $P < 0.05$

Example 8. Associations between glaucoma and gene

**polymorphisms of endothelin-1 and endothelin type A  
receptor**

**Purpose:** Endothelin 1 (ET-1), a potent vasoconstrictor, may affect regulation of intraocular pressure and ocular vessel  
5 tone. Thus, ET-1 and its receptors may contribute to development of glaucoma. We investigated whether gene polymorphisms of ET-1 (*EDN1*) and its receptors *ET<sub>A</sub>* (*EDNRA*) and *ET<sub>B</sub>* (*EDNRB*) were associated with glaucoma phenotypes and clinical features.

10 **Methods**

**Study population:**

A total of 650 Japanese subjects (224 normal controls, 176 POAG patients, and 250 NTG patients), recruited from seven Japanese medical institutions, were  
15 examined in this study. All subjects were unrelated. Mean age ( $\pm$  standard deviation) at diagnosis of OAG was  $57.2 \pm 12.8$  years. OAG subjects were divided into POAG patients and NTG patients, aged  $58.8 \pm 12.2$  and  $56.1 \pm 13.2$  years at diagnosis, respectively (Table 1). Mean age at the  
20 time of examination was  $70.0 \pm 11.2$  years in controls. We purposely selected older control subjects to reduce the likelihood that a subset of controls would later develop glaucoma.

Ophthalmic examinations included slit-lamp

biomicroscopy, optic disc examination, IOP measurement by Goldmann applanation tonometry, and gonioscopy. Visual fields were assessed with Humphrey automated perimetry (program 30-2) or Goldmann perimetry. Severity of visual field defects was scored from 1 to 5. Data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. This severity scale followed Kozaki's classification, which has been used most widely in Japan so far, based on Goldmann perimetry, or by the classification established for the Humphrey Field Analyzer.

POAG was diagnosed on fulfillment of all of the following criteria: maximum IOP was above 21 mm Hg; open angles on gonioscopy; typical glaucomatous disc cupping associated with visual field changes; and absence of other ocular, rhinologic, neurological, or systemic disorders potentially causing optic nerve damage. We excluded patients with elevated IOP secondary to defined causes (e.g., trauma, uveitis, steroid administration, or exfoliative, pigmentary, or neovascular glaucoma). POAG patients with MYOC mutations and JOAG patients were also excluded. NTG was diagnosed by the same criteria as POAG except that IOP did not exceed 21 mm Hg at all times during

the follow-up period. Normal control subjects had IOP less than 20 mm Hg, no glaucomatous disc changes, and no family history of glaucoma.

**DNA extraction and genotyping of the polymorphisms**

5           Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Nine single nucleotide polymorphisms (SNPs) were detected among all participants: four for *EDN1* (T-1370G, +138/ex1 del/ins, G8002A, K198N); four for *EDNRA* (G-231A, H323H, C+70G, C+1222T); and one for  
10 *EDNRB* (L277L). These polymorphisms are listed at <http://genecanvas.idf.inserm.fr/>. We genotyped these SNPs using the Invader<sup>®</sup> assay (Third Wave Technologies, Inc, Madison, WI), which was recently developed for high-throughput genotyping of SNPs (Lyamichev V et. al., Nat  
15 Biotechnol 1999;17:292-296, the contents of the cited reference are herein incorporated by reference).

The oligonucleotide sequences of primary probes and Invader<sup>®</sup> probes used in this study are listed in Table 20.

[illegible]

Nine polymorphisms were detected among all participants. These polymorphisms are listed at <http://genecanvar.nl/index.html>. Genotyping of the polymorphisms were performed by the Invader® assay using the probes listed above.



### Statistical analysis

Comparisons of genotype distributions in normal controls with those in OAG patients, POAG patients, and NTG patients were performed by  $\chi^2$  analysis. Associations of clinical characteristics (age at diagnosis, untreated maximum of IOP, and visual field score at diagnosis) with genotypes were assessed by the Mann-Whitney *U* test. Statistical analyses were carried out with SPSS for Windows (version 12.0; SPSS Inc, Chicago, IL). A value of  $p < 0.05$  was considered to be significant.

### Results

Table 21 shows genotype and allele frequencies obtained in this study. Distributions were consistent with Hardy-Weinberg equilibrium. For the *EDN1*/+138/ex1 del/ins polymorphism, frequencies of the del/del and del/ins + ins/ins genotypes respectively were 74.2% and 25.8% in OAG patients overall ( $p = 0.016$ ), 74.4% and 25.6% in POAG patients ( $p = 0.047$ ), and 74.0% and 26.0% in NTG patients ( $p = 0.037$ ), compared with 65.2% and 34.8% in control subjects. For the *EDN1*/K198N polymorphism, 53.2% of OAG patients were found to have the KK genotype, which was significantly higher than the 43.8% prevalence in control subjects ( $p = 0.022$ ). When OAG patients were divided into those with POAG and those with NTG, frequency of the KK

genotype in NTG patients was much higher than in controls (p=0.008), while genotype and allele frequency distributions in POAG patients did not differ statistically from those in controls. A gender difference was noted; specifically, the KK genotype was significantly more prevalent in female NTG patients (p=0.010 vs. female controls) than in male NTG patients (p=0.251 vs. male controls; Table 22). Polymorphism of *EDN1*/G8002A in the intron 4 region was highly coincident with *EDN1*/K198N, except in one sample (data not shown).

Frequencies of *EDNRA*/C+1222T genotypes (CC vs. CT+TT) differed slightly between OAG patients and controls (p=0.036). Distribution of genotypes for other polymorphisms showed no significant differences between any patient group and controls.

Characteristics of patients are examined in dominant model and recessive model of each polymorphism, and data with significant differences are shown in Table 23. In OAG patients overall and in POAG patients, no characteristic showed a significant difference between genotype groups. In NTG patients, however, the AA group of *EDNRA*/G-231A had poorer visual field scores at diagnosis than the GG+GA group (3.0±0.8 vs. 2.7±0.6, p=0.043). We also found significantly poorer visual field scores at diagnosis in

the GG group for *EDNRA*/C+70G than the CC+CG group among NTG patients ( $3.0 \pm 0.7$  vs.  $2.7 \pm 0.7$ ,  $p=0.014$ ). Untreated maximum of IOP in the TT group for *EDNRA*/H323H was statistically higher than in the CC+CT group in NTG patients ( $17.2 \pm 2.2$  vs. 5  $16.6 \pm 2.3$ ,  $p=0.040$ ). Other polymorphisms in NTG patients showed no significant differences in characteristics between genotype groups.

Table 21. Genotype and allele frequencies of EDN1, EDNRA, and EDNRB polymorphisms in control subjects and glaucoma patients

Polymorphism		Genotype frequency		p value	Allele frequency		p value
		TT	TG+GG		T	G	
EDN1/T-1370G	Control (n=224)	133 (59.4)	91 (40.6)		350 (78.1)	98 (21.9)	
	OAG (n=426)	273 (64.1)	153 (35.9)	0.239	675 (79.2)	177 (20.8)	0.844
	POAG (n=176)	108 (61.4)	68 (38.6)	0.687	275 (78.1)	77 (21.9)	1.000
	NTG (n=250)	165 (66.0)	85 (34.0)	0.136	400 (80.0)	100 (20.0)	0.478
		del del	del ins + ins ins		del	ins	
EDN1/+138/ex1 del/ins	Control (n=224)	146 (65.2)	78 (34.8)		364 (81.3)	84 (18.8)	
	OAG (n=426)	316 (74.2)	110 (25.8)	0.016*	724 (88.2)	118 (13.8)	0.020*
	POAG (n=176)	131 (74.4)	45 (25.6)	0.047*	303 (86.1)	49 (13.9)	0.069
	NTG (n=250)	185 (74.0)	65 (26.0)	0.037*	431 (88.2)	69 (13.8)	0.039*
		KK	KN+NN		K	N	
EDN1/K198N	Control (n=224)	98 (43.8)	126 (56.3)		295 (65.8)	153 (34.2)	
	OAG (n=426)	226 (53.2)	199 (46.8)	0.022*	609 (71.6)	241 (28.4)	0.031*
	POAG (n=176)	86 (49.1)	89 (50.9)	0.284	245 (70.0)	105 (30.0)	0.213
	NTG (n=250)	140 (56.0)	110 (44.0)	0.008*	364 (72.8)	136 (27.2)	0.020*
		GG	GA+AA		G	A	
EDNRA/G-231A	Control (n=224)	82 (27.7)	162 (72.3)		244 (54.5)	204 (45.5)	
	OAG (n=426)	118 (27.8)	307 (72.2)	0.981	455 (53.5)	395 (46.5)	0.748
	POAG (n=176)	52 (29.5)	124 (70.5)	0.681	195 (55.4)	157 (44.6)	0.792
	NTG (n=249)	66 (26.5)	183 (73.5)	0.774	280 (52.2)	238 (47.8)	0.488
		TT	TC+CC		T	C	
EDNRA/H323H	Control (n=224)	122 (54.5)	102 (45.5)		327 (73.0)	121 (27.0)	
	OAG (n=426)	228 (53.5)	198 (46.5)	0.819	626 (73.6)	228 (26.5)	0.852
	POAG (n=176)	95 (54.0)	81 (46.0)	0.923	259 (73.6)	93 (26.4)	0.852
	NTG (n=250)	133 (53.2)	117 (46.8)	0.783	387 (73.4)	133 (26.6)	0.887
		CC	CG+GG		C	G	
EDNRA/C+70G	Control (n=224)	51 (27.2)	163 (72.8)		220 (51.1)	219 (48.9)	
	OAG (n=426)	128 (30.0)	298 (70.0)	0.453	462 (54.2)	390 (45.8)	0.286
	POAG (n=176)	57 (32.4)	119 (67.6)	0.262	198 (55.7)	156 (44.3)	0.199
	NTG (n=250)	71 (28.4)	179 (71.6)	0.777	286 (53.2)	234 (46.8)	0.521
		CC	CT+TT		C	T	
EDNRA/C+1222T	Control (n=224)	137 (61.2)	87 (38.8)		347 (77.5)	101 (22.5)	
	OAG (n=426)	224 (52.6)	202 (47.4)	0.036*	620 (72.8)	232 (27.2)	0.066
	POAG (n=176)	92 (52.3)	84 (47.4)	0.074	254 (72.2)	98 (27.8)	0.085
	NTG (n=250)	132 (52.8)	118 (47.2)	0.057	366 (73.2)	134 (26.8)	0.130
		AA	AG+GG		A	G	
EDNRB/L277L	Control (n=224)	77 (34.4)	147 (65.6)		254 (56.7)	194 (43.3)	
	OAG (n=426)	118 (27.8)	307 (72.2)	0.081	443 (52.1)	407 (47.9)	0.116
	POAG (n=176)	48 (27.3)	128 (72.7)	0.128	184 (52.3)	188 (47.7)	0.212
	NTG (n=249)	70 (28.1)	179 (71.9)	0.142	299 (52.0)	239 (48.0)	0.148

Data are n (%).

\*  $P < 0.05$  ( $\chi^2$  test).

Genotype distributions showed significant differences for EDN1/+138/ex1 del/ins ( $p=0.016$ ) and EDN1/K198N ( $p=0.022$ ) polymorphisms, and a slight difference for EDNRA/C+1222T polymorphism ( $p=0.036$ ) between OAG patients and controls. After dividing the OAG group into POAG and NTG, frequency of the KK genotype for the EDN1/K198N polymorphism in NTG patients was much higher than in controls ( $p=0.008$ ).

Table 22. Genotype frequency of EDN1/K198N polymorphism  
in male and female subjects

Polymorphism	Male		Female					
	Genotype	frequency	p value	Genotype	frequency	p value		
EDN1/K198N	KK	KN+NN		KK	KN+NN			
	Control (n=100)	48 (48.0)	54 (54.0)	Control (n=124)	52 (41.9)	72 (58.1)		
	OAG (n=218)	112 (51.4)	106 (48.6)	0.373	OAG (n=207)	114 (55.1)	93 (44.9)	0.021*
	POAG (n=89)	43 (48.5)	51 (51.5)	0.726	POAG (n=76)	38 (50.0)	38 (50.0)	0.266
	NTG (n=119)	64 (53.8)	55 (46.2)	0.251	NTG (n=131)	76 (58.0)	55 (42.0)	0.010*

Data are n (%).

\*  $P < 0.05$  ( $\chi^2$  test).

In the EDN1/K198N polymorphism, genotype distributions differed according to gender. The KK genotype for this polymorphism was significantly more prevalent in female NTG patients ( $p=0.010$  vs. female controls) than in male NTG patients ( $p=0.251$  vs. male controls).

Table 23. Characteristics of glaucoma patients according to genotype

Polymorphism	Type of glaucoma	Characteristic	Genotype	p value
EDMRA/C-231A	NTG		GG+GA	AA
		Age at diagnosis (years)	58.9±131 (n=182)	53.6±135 (n=56)
		Untreated maximum IOP (mm Hg)	17.1±2.3 (n=189)	16.4±2.2 (n=52)
		Visual field score at diagnosis	2.7±0.6 (n=194)	3.0±0.8 (n=56)
EDMRA/H23H	NTG		TT	TC+CC
		Age at diagnosis (years)	55.7±135 (n=131)	56.6±12.9 (n=117)
		Untreated maximum IOP (mm Hg)	17.2±2.2 (n=129)	16.6±2.3 (n=112)
		Visual field score at diagnosis	2.8±0.7 (n=133)	2.7±0.7 (n=117)
EDMRA/C+T03	NTG		CC+CG	GG
		Age at diagnosis (years)	55.7±133 (n=194)	51.8±12.7 (n=54)
		Untreated maximum IOP (mm Hg)	17.0±2.2 (n=188)	16.5±2.3 (n=53)
		Visual field score at diagnosis	2.7±0.7 (n=195)	3.0±0.7 (n=53)

Data are means ± SD.

\*  $P < 0.05$  (Mann-Whitney U test).

The AA genotype of EDMRA/C-231A and the GG genotypes of EDMRA/C+T03 were associated with worse visual field defects in NTG patients ( $p=0.043$  and  $0.014$ , respectively). The EDMRA/H23H polymorphism influenced untreated maximum IOP among NTG patients ( $p=0.040$ ).

In male subjects, the following correlations were confirmed:

- 1) The A138insertion/deletion(A138I/D) polymorphism in exon

1 of the Endothelin-1 gene is associated with both of POAG and NTG (Table 24).

2)The -231A>G polymorphism of promoter region of the Endothelin receptor A gene is associated with NTG,  
5 especially with patients with intraocular pressure at less than 15mmHg (Table 25).

3)The CAC to CAT substitution at codon No. 233 in exon 6 of the Endothelin receptor A gene (His323His) is associated with NTG, especially with patients with intraocular  
10 pressure at less than 15mmHg (Table 26).

4)The CTG to CTA substitution at codon No. 277 in exon 4 of the Endothelin receptor B gene is associated with both of POAG and NTG (Table 27).

In female patients, following correlations were  
15 confirmed:

1)The AAG to AAT substitution at codon No. 198 of the endothelin-1 gene (Lys198Asn) is associated with NTG (Table 28).

2)The -1370T>G polymorphism of the Endothelin-1 gene promoter region is associated with NTG (Table 29).  
20

3)The +70C>G(70 bases from the stop codon) polymorphism in 3' non-coding region of the Endothelin receptor A is associated with POAG (Table 30).

4)The +1222C>T(1222 bases from the stop codon) polymorphism

in 3' non-coding region of the Endothelin receptor A is associated NTG (wherein the intraocular pressure is 16mmHg-21mmHg) (Table 31).

**Table 24. Endothelin A138I/D (Male)**

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		I/I	I/D	D/D		I/I	I/D+D/D		I/I+I/D	D/D	
Cont rol	100	4	34	62		4	96		38	62	
FOAG	100	3	21	76		3	97		24	76	0.032
NTG	119	1	28	90		1	118		29	90	0.029

5

**Table 25. Endothelin Receptor A -231A>G (Male)**

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		AA	AG	GG		AA	AG+GG		AA+AG	GG	
Cont rol	100	22	45	33		22	78		67	33	
FOAG	100	24	51	25		24	76		75	25	
NTG	119	30	60	29		30	89		90	29	
H-NTG	89	17	45	27		17	72		62	27	
L-NTG	25	11	12	2	0.017	11	14	0.026	23	2	0.025

H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: NTG patients with maximal intraocular pressure at 15mmHg or less.

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**Table 26. Endothelin Receptor A H323H C>T His323His (Male)**

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Cont rol	100	9	40	51		9	91		49	51	
FOAG	100	7	38	55		7	93		45	55	
NTG	119	11	50	58		11	108		61	58	
H-NTG	89	7	32	50		7	82		39	50	
L-NTG	25	4	14	7		4	21		18	7	0.039



H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: MTG patients with maximal intraocular pressure at 15mmHg or less.

5

Table 27. Endothelin Receptor B L277L G>A Leu277Leu (Male)

	n	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		GG	GA	AA		GG	GA+AA		GG+GA	AA	
Cont rol	100	18	41	41		18	82		59	41	
POAG	100	26	48	26		26	74		74	26	0.025
NTG	119	26	61	32		26	93		87	32	0.027

Table 28. Endothelin Lys198Asn G>T or K198N (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		KK	KN	NN		KK	KN+NN		KK+KN	NN	
Cont rol	124	52	59	13		52	72		111	13	
POAG	76	38	33	5		38	38		71	5	
NTG	131	76	38	17	0.009	76	55	0.010	114	17	

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Table 29. Endothelin -1370T>G (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		TT	TG	GG		TT	TG+GG		TT+TG	GG	
Cont rol	124	66	56	2		66	58		122	2	
POAG	76	49	24	3		49	27		73	3	
NTG	131	84	39	8	0.013	84	47		123	8	

Table 30. Endothelin Receptor A +70C&gt;G (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		CC	CG	GG		CC	CG+GG		CC+CG	GG	
Control	124	29	59	36		29	95		88	36	
POAG	76	28	32	16		28	48	0.041	60	16	
NTG	131	35	66	30		35	96		101	30	

Table 31. Endothelin Receptor A +1222C&gt;T (Female)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Control	124	74	42	8		74	50		116	8	
POAG	76	40	30	6		40	36		70	6	
NTG	131	66	54	11		66	65		120	11	
H-NTG	92	42	42	8		42	50	0.041	84	8	
L-NTG	35	21	11	3		21	14		32	3	

5 H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.

L-NTG: MTG patients with maximal intraocular pressure at 15mmHg or less.

Partial nucleotide sequences of endothelin-1(EDN1) and  
10 endothelin receptor A (EDNRA) and endothelin receptor B (EDNRB) comprising the targeted polymorphisms are shown below

EDN1 -1370 (underlined) T>G

2101 ttgaattcca ccctecatcc ccagaaaaac tggagtaaaa caaaaagagg agatggacaa  
15 2161 agtgtgtatt tgaatggcatc ccctgggaag agactctaaa tttatcccat aggtcttact  
2221 gggccactgt gaggcgtttg gtggagaaca aacaaaaatt ctgggtgtctc agttgtctaa  
2281 cctgaaaaat gggactagcy gaaaaagcca atgtgttcca tgcacctttt gctttcttta

2341 ttaaggcatg atgtcacctg tacagtaact gccctgtgtg tacttcaggg (SEQ ID  
NO:142)

END1 +138 (underlined) ins/del (each one of the a at 3743-3745)

5 3661 ccagctctcc accgcccggt gcgcctgcag acgctccgct cgtgccttc tctcctggca  
3721 ggcgctgcct tttctcccg ttaaaggga cttgggctga aggatcgctt tgagatctga  
3781 ggaacccgca gcgctttgag ggacctgaag ctgtttttct tcgttttctt ttgggttcag  
3841 ttgaaacggg aggtttttga tccctttttt tcagaatgga ttattgtctc atgattttct  
(SEQ ID NO:143)

10 (atg is the initiation codon)

EDNRA +70 (underlined) C>G

63601 atccagtga agaaccagca tcaaaacaac cacaacacag accggagcag ccataaggac  
63661 agcatgaacgaccaccctt agaagcactc ctccgtactc ccataatcct ctccggagaaa  
15 63721 aaatcacaa ggcaactgtg agtccgggaa tctcttctct gatccttctt ccttaattca  
63781 ctcccacacc caagaagaaa tgctttccaa aaccgcaagg gtagactggt ttatccacce  
63841 acaacatcta cgaatcgtao ttctttaatt gatctaattt acatattctg cgtgtgtgat (SEQ  
ID NO:144)

(tga is the translation termination codon)

20

EDNRA +1222 (underlined) C>T

64741 ttaatttttc ttaaaatgtt aactggcagt aagtcttttt tgatcattcc cttttccata  
64801 taggaacatc aattttgaag tggccagatg agtttatcat gtcagtgaac aataattacc  
64861 cacaatgcc accagaactt aacgattctt cacttcttgg gggtttcagt atgaacctaa  
25 64921 ctcccacccc caacatctcc ctcccacatt gtcaccattt caaaggcccc acagtgaact

64981 ttgctgggca ttttccaga tgttacaga ctgtgagtac agcagaaaat cttttactag

(SEQ ID NO:145)

**EDNRA codon No. 323 (underlined) (T>C) His323His**

5 60721 gaggtagagg cagtgaagc caggctgttc tctggctct tctttgaatt attctttctc  
60781 tgggtgtctgc tacttcttgg tactgtagtt ctgcatcta gtataaaaac actaaatttg  
60841 ttgtcctatt tttttctcac ttctcttag cgtcgagaag tggcaaaaac agttttctgc  
60901 ttggttgtaa tttttgtctt ttgctgggtc cctcttcatt taagccgtat attgaagaaa  
60961 actgtgtata acgagatgga caagaaccga tgtgaattac ttaggtatga tctgtgttac  
10 61021 tcgctagaaa attggagttt ctacagtttt catatttata atacttttac aaaaccagct

(SEQ ID NO:146)

**EDNRA -231(underlined) A>G**

2041 ggaggagacg gggaggacag actggaggcg tgctcctccg gagttttctt tttcgtgcga  
15 2101 gccctcgcgc gcgcgtacag tcaccccgct ggtctgacga ttgtggagag gcggtggaga  
2161 ggcttcaccc atcccccccg gtcgtgcgcg gggattgggg tcccacgcag acctccccgg  
2221 gagaagcagt gcccaggagg ttttctgaag ccggggaagc tgtgcagccg aagccgccgc  
2281 cgcgccggag cccgggacac cgccaccct ccgcgccacc caccctcgcc ggctccgggt  
2341 tctcttgccc caggcgcgcg gcggaccgcg cagctgtctg cgcacgcga gctccacggt

20 (SEQ ID NO:147)

**EDNRB codon No. 277(underlined) Leu277Leu (CTG to CTA)**

75361 taatcattcc ctgatgaatt tttttaagtt taacatttgt tatataagat tttcttacag  
75421 aggagtatta atcgtazaaa ttctctcacc cctatagttt tacaagacag caaaagattg  
25 75481 gtggcctgttc agtttctatt tctgcttgcc attggccact actgcatttt ttatatacat

75541 aatgacctgt gaaatgttga gaaagaaaag tggcatgcag attgctttaa atgataacct

75601 aaagcaggta agaaaataca aatatttgat aactcgtggt tgaattata attatgaata

(SEQ ID NO:148)

## 5 Example 9. Association between Gene Polymorphism of $\beta 1$ adrenergic receptor (ADRB1) and Glaucoma

### Methods

Association between gene polymorphism of ADRB1 and  
glaucoma was examined among POAG, NTG patients and normal  
10 (control) subjects using PCR-RFLP techniques (Table 32-1).

Table 32-1. Primer sequences

Gene		Primer sequences		Restriction Enzyme
ADRB1	F	CCG CCT CTT CGT CTT CTT CAA CTG	Bsm FI	(SEQ ID NO:149)
Gly389Arg	R	GAT AGC AGG TGA ACT CGA AGC CCA		(SEQ ID NO:150)

## 15 Results

As shown in Table 32-2, the polymorphism of  
Gly389Arg in ADRB1 is associated with NTG (Table 32-2).

Table 32-2.  $\beta 1$ -Adrenalin Receptor Gly389Arg

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		CC	CG	GG		CC	CG+GG		CC+CG	GG	
Control	240	147	78	15		147	93		225	15	
POAG	191	127	58	6		127	64		185	6	
NTG	284	197	80	7	0.038	197	87		277	7	0.031

Partial nucleotide sequence of  $\beta$ 1-Adrenalin Receptor  
comprising the targeted polymorphism.

B1AR codon 389 (underlined GGA(Gly) to CGA(Arg) Gly389Arg

1021 ttctctggcca acgtggtgaa ggccttccac cgcgagctgg tgcccgaccg cctcttctgc

5 1081 ttcttcaact ggctgggcta cgccaactcg gccttcaacc ccatcatcta ctgccgcagc

1141 ccgcacttcc gcaaggcctt ccagggactg ctctgctgcg cgcgcagggc tgcccgcggg

1201 cgccacgcga cccacggaga cgggccgcgc gctcgggct gtctggcccg gcccgacc

1261 ccgccatcgc cggggccgc ctcgacgac gacgacgacg atgtcgtcgg ggcacgccc

(SEQ ID NO:151)

10

#### Example 10. Correlation between Gene Polymorphism of E- Selectin and glaucoma

##### Methods

Relationship between a E-selectin gene polymorphism and  
15 glaucoma among subject with POAG, NTG and normal subject  
was examined by means of Invader<sup>®</sup> method.

Invader<sup>®</sup> oligonucleotides (Invader<sup>®</sup> probe) used to  
detect the C/T polymorphism of SELE gene are shown in Table  
33-1.

20

Table 33-1

Mutation	nucleotide change	Target	Probe	Sequence	Length (bp)	T <sub>m</sub> (°C)	Dye	(SEQ ID NO:152)
SELE 1402 CT	G to T	Anti-sense	Wild	Flar-CATGGATCAAGTCAAGTTGA	32	83.8	RED	(SEQ ID NO:152)
			Mutant	Flar-TATGGATCAAGTCAAGTTGAG	31	83.4	FAM	(SEQ ID NO:153)
			Invader	TGTTGTCGCTTCAGGCTGTGAGGAGGGATTGATTAA	37	77.2		(SEQ ID NO:154)

**Results**

The 1402C>T polymorphism of E-selectin gene was confirmed being associated with both of POAG and NTG.

Table 33-2) .

5

**Table 33-2. E-selectin 1402C>T**

	N	Genotype Frequency			p	Genotype Frequency		P	Genotype Frequency		$\chi^2$ test p
		CC	CT	TT		CC	CT+TT		CC+CT	TT	
Control	224	138	67	19		138	86		205	19	
POAG	250	150	90	10		150	100		240	10	0.042
NTG	176	117	53	6		117	59		170	6	0.037

Partial nucleotide sequence of E-selectin comprising the targeted polymorphism is as follows:

10 **SELE No. 1402 (underlined) C>T**

7561 tgtttttatt ttattttaag ataaaaagaa ctattgaaga gcttgggaac ttggttacct

7621 tgggaaacgt attgctggag atgcaaaca acttctaaag tgctctctcg tgtgttcag

7681 ctgtgagatg cgatgctgtc caccagcccc cgaaggggtt ggtgagggtg gctcattccc

7741 ctattggaga attcacctac aagtcctctt gtgccttcag ctgtgaggag ggatttgaat

15 7801 tacatggatc aactcaactt gagtgcacat ctcagggaca atggacagaa gaggttcctt

7861 cctgccaaag tagaattgag tgcagacttt tttagggtag aggtcaaata cttcataaag

7921 tttctgaacc tagattgcc caaaggggtt tggctcctaatt ttctacatg ctgaaaaacta

7981 agtagcgctt acactttaca ttcatgtgtg acttttaagc aagttttgga agttttccag

8041 tagatttttc tgaaaactctg cctgtgtacc taacatttgc agtggtaaaa tgttcaagcc

20 8101 tggcagttcc gggaaagatc aacatgagct gcagtgggga gcccggtgtt ggcactgtgt

(SEQ ID NO:155)



**Example 11. Paraoxonase 1 gene polymorphisms are associated with clinical features of open-angle glaucoma**

**Purpose:** Oxidative derivatives of low-density lipoprotein (LDL) are injurious to endothelium. Endothelial dysfunction  
5 is known to be involved in the pathogenesis of open-angle glaucoma (OAG). High-density lipoprotein (HDL) prevents the oxidative modification of LDL. We examined whether polymorphisms in the paraoxonase 1 (PON1), PON2, and platelet-activating factor acetylhydrolase (PAF-AH) genes,  
10 HDL-associated antioxidant enzymes, were associated with OAG in a Japanese population.

**MATERIALS and METHODS**

**Patients and control study subjects**

Six hundred and ninety-eight blood samples were  
15 collected at seven Japanese institutions. Subjects included 190 POAG patients, 268 NTG patients, and 240 normal controls. None subject was related to any other.

Age at the blood sampling (mean  $\pm$  SD) was  $65.3 \pm 11.9$  years in POAG patients,  $58.8 \pm 13.4$  years in NTG  
20 patients, and  $69.7 \pm 11.2$  years in normal subjects, normal control subjects were significantly older than POAG patients ( $p < 0.001$ ) or NTG patients ( $p < 0.001$ ), which would reduce the likelihood of control subjects eventually developing glaucoma.

Clinical features recorded in glaucoma patients were age at diagnosis, IOP at diagnosis, and visual field defects at diagnosis. Severity of visual field defects was scored from 1 to 5. Data obtained with different perimeters were combined using a five-point scale defined as follows:

1 = no alternation; 2 = early defect; 3 = moderate defect; 4 = severe defect; 5 = light perception only or no vision. Field defects were judged to be early, moderate, or severe according to Kozaki's classification based on Goldmann perimetry or by the classification used for the Humphrey field analyzer. The former classification has been most widely used in Japan so far.

All patients received serial ophthalmic examinations including IOP measurements by Goldmann applanation tonometry, Humphrey perimetry (30-2) or Goldmann perimetry, gonioscopy, and optic disc examination including fundus photograph. All of glaucoma patients were diagnosed according to the following criteria: the presence of typical optic disc damage with glaucomatous cupping (cup/disc ratio  $>0.7$ ) and loss of neuroretinal rim; reproducible visual field defects compatible with the glaucomatous cupping; and open angles on gonioscopy. Among the OAG patients, POAG was diagnosed if they had an IOP  $>21$  mm Hg at any time during the follow-up period. Patients

with exfoliative glaucoma, pigmentary glaucoma, and corticosteroid-induced glaucoma were excluded. Among the OAG patients, NTG was diagnosed when: the untreated peak IOP was consistently equal to or less than 21 mm Hg at all  
5 times including the 3 baseline measurements and that during the diurnal testing values (every 3 hours from 6 AM to 24 PM); the peak IOP with or without medication after diagnosis was consistently <22 mm Hg throughout the follow-up period; and the absence of a secondary cause for  
10 glaucomatous optic neuropathy, such as a previously elevated IOP following trauma, a period of steroid administration, or uveitis.

Control subjects were recruited from among Japanese individuals who had no known eye abnormalities except for  
15 cataracts. These subjects numbered 196 and were older than 40 years, with IOP below 20 mm Hg, no glaucomatous disc change, and no family history of glaucoma.

### Genotyping

Genomic DNA was isolated from peripheral blood  
20 lymphocytes by standard methods. Four SNPs were then detected in all participants: two for *PON1* (L55M, Q192R); one for *PON2* (Cys311Ser, C311S); and one for *PAF-AH* (V279F).

These SNPs were genotyped by means of the Invader<sup>®</sup> assay (Third Wave Technologies, Inc, Madison, WI, USA)

which was recently developed for high-throughput genotyping of SNPs. The oligonucleotide sequences of primary probes and Invader® probes used in this study were listed in Table 34.

Table 34. Sequences of primary probes and Invader oligonucleotides used in assays

Polymorphism	Nucleotide change	Target	Probe	Probe	Sequence
PON M5L	A to T	Sense	Wild	A probe	Flap sequence+TGCTTCAGAGCCAGT (SEQ ID NO:154)
		Antisense	Mutant	T probe	Flap sequence+AGTCTTCAGAGCCAGT (SEQ ID NO:157)
		Invader	Invader	Invader	Flap sequence+AGTCTTCAGAGCCAGT (SEQ ID NO:159)
PON Q128	A to G	Sense	Wild	A probe	Flap sequence+GATCTGGGAGATGATG (SEQ ID NO:158)
		Antisense	Mutant	G probe	Flap sequence+GATCTGGGAGATGATG (SEQ ID NO:160)
		Invader	Invader	Invader	Flap sequence+GATCTGGGAGATGATG (SEQ ID NO:161)
PAE-AH V29F	G to T	Sense	Wild	A probe	Flap sequence+AGCATTCTATGACAAATGATCATTTTCTGACCCCTACTACT (SEQ ID NO:162)
		Antisense	Mutant	T probe	Flap sequence+AGCATTCTATGACAAATGATCATTTTCTGACCCCTACTACT (SEQ ID NO:163)
		Invader	Invader	Invader	Flap sequence+AGCATTCTATGACAAATGATCATTTTCTGACCCCTACTACT (SEQ ID NO:164)

### Statistical analysis

Hardy-Weinberg equilibrium was assessed by chi-squared analysis. Frequencies of the genotypes and alleles were compared between cases and controls by chi-squared analysis. Multivariate analyses were performed with a logistic regression model to confirm the association between the three clinical variables and the genotype. Comparison of IOPs between genotype groups of Q192R in the PON 1 gene was performed by Kruskal-Wallis test. Statistical analyses were carried out with SPSS (version 12.0; SPSS, Chicago, IL). A value of  $p < 0.05$  was considered to indicate significance.

### RESULTS

Distributions of genotypes for the four SNPs in glaucoma patients and controls are shown in Table 35. The L55M polymorphism of the PON1 gene had a significantly different genotype frequency in patients with NTG.

Distribution of genotypes for polymorphisms in the PON2 gene and PAF-AH gene showed no significant differences between any patient group and controls (Table 35). And there was no significant difference in allele frequency of the 4 SNPs.

Table 35. Genotype frequency of PON1, PON2, and PAF-AH polymorphisms  
in Japanese control subjects and glaucoma patients

	PON1/L5M			PON1/QU9R			PON2/C311S			PAF-AH/V279F		
	LI	LM	MM	QQ	QR	RR	CC	CS	SS	VV	VF	FF
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Principale Control (N=226)	150	34	0	32	105	85	10	74	140	153	62	9
	84.8	15.2	0.0	14.4	47.3	38.3	4.5	33.0	62.5	68.3	27.7	4.0
OAG (N=14)	145	29	0	22	74	78	3	73	100	255	115	14
	83.3	16.7	0.0	12.6	42.5	44.8	0.021	1.7	44.3	52.8	25.4	3.3
NTG (N=205)	224	19	3	44	100	102	9	35	154	121	46	4
	91.1	7.7	1.2	0.009	17.9	48.7	3.6	35.5	69.9	69.1	27.6	2.9
												DE24

The distributions of the combined two polymorphisms  
of the PON1 gene in OAG population are shown in Table 36.  
As clearly shown, methionine (M) at position 55 (M allele)

was rarely associated with arginine (R) at position 192 (R allele). Analysis confirmed a linkage disequilibrium between the polymorphisms giving rise to leucine (L) at position 55 and arginine (R) at position 192 ( $P < 0.001$ ).

Table 36. Distribution of genotypes defined by polymorphisms of PON1  
gene affecting amino acids at position 55 and 192

	Q192R			Q192R		
	QQ	QR	RR	Total	Non-Recarrier	
					L-carrier	Re-carrier
LL	72	221	265	558	95	544
LM	23	58	0	81	3	0
MM	3	0	0	3		
Total	98	279	265	642		

Characteristics of patients were examined in dominant and recessive models for each polymorphism. In the recessive model, no significant difference was seen in



three characteristics in patients with OAG for any polymorphisms. Significant differences with the dominant model of PON1 polymorphisms are shown in Tables 37 and 38. For L55M polymorphism in the PON1 gene in OAG patients, the

5 LL group (non-55M carriers) was significantly younger at diagnosis than the LM+MM group (55M carriers) ( $56.8 \pm 12.8$  years vs.  $60.1 \pm 11.4$ ,  $p=0.028$ ) (Table 37). This association was not observed in POAG patients, but in NTG patients ( $55.6 \pm 13.1$  years vs.  $63.7 \pm 9.6$ ,  $p=0.001$ ).

10 For Q192R polymorphism, untreated maximum IOPs at diagnosis were significantly higher in OAG patients with QR+RR group (192R carriers) ( $21.5 \pm 7.4$  mm Hg) than those with QQ group (non-192Rcarriers) ( $18.7 \pm 5.3$  mm Hg,  $P=0.006$ , Table 38). Untreated maximum IOPs were higher in 192R

15 carriers than in non-carriers among POAG patients ( $27.5 \pm 7.0$  mm Hg vs.  $24.0 \pm 4.9$  for POAG,  $p=0.049$ ) as well as among NTG patients ( $15.8 \pm 2.8$  mm Hg vs.  $16.7 \pm 2.4$  for NTG,  $p=0.030$ ).

Table 37 Clinical characteristics of NTG patients according to genotype of L55M in the PON1 gene

Phenotype	Clinical characteristics	Genotype		P value*
		LL	LM+MM	
OAG	Age at diagnosis (ys)	56.8 ± 12.8 (n = 473)	60.1 ± 11.4 (n = 62)	0.028
	IOP at diagnosis (mm Hg)	21.1 ± 7.2 (n = 409)	21.5 ± 6.1 (n = 58)	0.681
	Visual field score at diagnosis	2.9 ± 0.8 (n = 476)	3.0 ± 0.7 (n = 63)	0.899
POAG	Age at diagnosis (ys)	58.6 ± 12.2 (n = 199)	58.2 ± 12.3 (n = 34)	0.836
	IOP at diagnosis (mm Hg)	27.3 ± 7.1 (n = 170)	25.9 ± 4.8 (n = 31)	0.352
	Visual field score at diagnosis	3.9 ± 0.9 (n = 200)	3.0 ± 0.7 (n = 35)	0.475
NTG	Age at diagnosis (ys)	55.6 ± 13.1 (n = 274)	63.7 ± 9.6 (n = 28)	0.001
	IOP at diagnosis (mm Hg)	16.6 ± 2.5 (n = 239)	16.6 ± 2.7 (n = 27)	0.984
	Visual field score at diagnosis	2.8 ± 0.7 (n = 276)	2.9 ± 0.7 (n = 28)	0.343

P value\* with Logistic regression analyses

Table 38 Clinical characteristics of glaucoma patients according to genotype of Q192R in the PON1 gene

Phenotype	Clinical characteristics	Genotype		P value*
		QQ	QR+RR	
OAG	Age at diagnosis (ys)	56.2 ± 13.9 (n = 77)	57.5 ± 12.4 (n = 468)	0.974
	IOP at diagnosis (mm Hg)	18.7 ± 5.3 (n = 66)	21.5 ± 7.4 (n = 409)	0.006
	Visual field score at diagnosis	2.7 ± 0.7 (n = 77)	2.9 ± 0.8 (n = 472)	0.100
POAG	Age at diagnosis (ys)	55.2 ± 12.8 (n = 29)	58.9 ± 12.0 (n = 210)	0.259
	Untreated IOP at diagnosis (mm Hg)	24.0 ± 4.9 (n = 23)	27.5 ± 7.0 (n = 183)	0.049
	Visual field score at diagnosis	2.8 ± 0.7 (n = 29)	3.1 ± 0.9 (n = 212)	0.415
NTG	Age at diagnosis (ys)	56.8 ± 14.6 (n = 48)	56.4 ± 12.7 (n = 258)	0.395
	Untreated IOP at diagnosis (mm Hg)	15.8 ± 2.8 (n = 43)	16.7 ± 2.4 (n = 226)	0.030
	Visual field score at diagnosis	2.7 ± 0.7 (n = 48)	2.8 ± 0.7 (n = 260)	0.155

P value\* with Logistic regression analyses

The Gly192Arg (Q192R) polymorphism in PON1 gene was associated with POAG (Table 39). The Leu55Met polymorphism was associated with NTG, especially with less than 15mmHg (Table 40)

Table 39 PON1 Gln192Arg (Q192R)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		QQ	QR	RR		QQ	QR+RR		QQ+QR	RR	
Control	224	32	107	85		32	192		139	85	
POAG	110	14	39	57	0.049	14	96	0.021	53	57	0.016
NTG	160	32	66	62		32	128		98	62	

Table 40 PON1 Leu55Met (L55M)

	N	Genotype Frequency			p	Genotype Frequency		p	Genotype Frequency		$\chi^2$ test p
		LL	LM	MM		LL	LM+MM		LL+LM	MM	
Control	226	192	34	0		192	34		226	0	
POAG	110	97	13	0		97	13		110	0	
NTG	160	144	13	3	0.013	144	16		157	3	
H-NTG	122	111	10	1		111	11		121	1	
L-NTG	34	29	3	2	0.034	29	5		32	2	0.009

- 5 H-NTG: NTG patients with intraocular pressure at 16 mmHg-21mmHg.
- L-NTG: MTG patients with maximal intraocular pressure at 15mmHg or less.
- 10 Conclusion: PON1 gene polymorphisms may influence features of Japanese patients with OAG, especially those with NTG.

Partial nucleotide sequence of Paraoxonase 1 gene containing the targeted polymorphisms is as follows:

PON1 Codon 55(underlined) TTG(Leu) to ATG(Met) (Leu55Met)  
and

PON1 Codon 192(underlined) CAA(Gln) to CGA(Arg) (Gln192Arg)

1 agagcctcct agcccgtcgg tgtctgcgcc cctcgatccc tttgtctatc cccgaccatg  
5 61 gcgaagctga ttgcgctcac cctcttgggg atgggactgg cactcttcag gaaccaccag  
121 tcttcttacc aaacacgact taatgtcttc cgagaggtag aaccgtaga acttcttaac  
181 tgtaatttag ttaaaggaat cgaaactggc tctgaagact tggagatact gcctaattgga  
241 ctggctttca ttagctctgg attaaagtat cctggaataa agagcttcaa cccaacagt  
301 cctggaaaaa tacttctgat ggacctgaat gaagaagatc caacagtgtt ggaattgggg  
10 361 atcactggaa gtaaatttga tgtatcttca tttaccctc atgggattag cacattcaca  
421 gatgaagata atgccatgta cctcctgggtg gtgaaccatc cagatgccaa gtccacagtg  
481 gagttgttta aatttcaaga agaagaaaaa tgccttttgc atctaaaaac catcagacat  
541 aaacttctgc ctaatttgaa tgatattgtt gctgtgggac ctgagcactt ttatggcaca  
601 aatgatcact attttcttga cccctactta caatcctggg agatgtattt gggtttagcg  
15 661 tggtcgtatg ttgtctacta tagtccaagt gaagttcgag tgggtggcaga aggattttag  
721 tttgtctaag gaatcaacat ttcacccgat ggcaagtatg tctatatagc tgagtgtctg  
781 gctcataaga ttcattgtga tgaaaagcat gctaatttga ctttaactcc attgaagtcc  
841 cttgacttta ataccctcgt ggataacata tctgtggatc ctgagacagg agacctttgg  
901 gttggatgcc atcccaatgg catgaaaatc ttcttctatg actcagagaa tcctcctgca  
20 961 tcagaggtgc ttcgaatcca gaacattcta acagaagaac ctaaaagtac acaggtttat  
(SEQ ID NO:165)

Example 12. Evaluation of the Noelin 2 gene in the  
ethiology of open-angle glaucoma

**Purpose:** To screen for mutations in the *Noelin 2* gene in Japanese patients with open-angle glaucoma using denaturing high-performance liquid chromatography (DHPLC).

**Methods**

5    ***Subjects***

A total of 616 blood samples were collected at eight institutions in Japan. There were 276 POAG patients, 340 NTG patients, and 300 normal controls, and none of the subjects was related to others in this study.

10   **DNA Extraction and PCR Conditions**

All of the blood samples were analyzed at Keio University. Genomic DNA was isolated from peripheral blood lymphocytes by phenol-chloroform extraction. The 6 exonic coding regions of the *Noelin 2* gene were amplified by  
15   polymerase chain reaction (PCR) using the primer sets listed in Table 41.

**Table 41. Primer sequences, PCR product sizes, and PCR annealing and DHPLC analysis temperatures**

Exon	Primer Sequences (5' to 3')	PCR product size (bp)	PCR T <sub>m</sub> (°C)	DHPLC T <sub>m</sub> (°C)	
1	F not determined R not determined				
2	F GCGAGACCCCTCACTGGGATT R GCCTGGAGAGGAGCTGGATT	344	67	62.0, 63.0, 64.0	(SEQ ID NO:166) (SEQ ID NO:167)
3	F GGTGGGATTGGGGAAGGA R CCAAGACATGACTCCATTGTAGGAA	284	67	60.3, 62.3, 64.3	(SEQ ID NO:168) (SEQ ID NO:169)
4	F GAGTCAGAGGTGTGAGTCATGT A R CCGTTGCTGCAGGTCTCTATA	249	65	62.7, 63.2, 63.7	(SEQ ID NO:170) (SEQ ID NO:171)
4	F CAGACACGCGGACCATTTGTA B R GGTGTGGCAGTCAGAGATCA	208	65	63.1, 64.1, 65.1	(SEQ ID NO:172) (SEQ ID NO:173)
5	F CCCAACTTGATCACAGCACTT R CTAGGCACCTATGGGCAGTCAA	269	65	61.7, 63.7, 64.7	(SEQ ID NO:174) (SEQ ID NO:175)
6	F CTAATGGCTGTAGCTGGTGCT A R GTAGGGGAAGGTGTTGTGTAA	336	65	62.5, 63.5, 64.5	(SEQ ID NO:176) (SEQ ID NO:177)
6	F CCAGAGCAACGTGGTGGTCA B R GGTAGCCGGTGTCCACAGGA	248	67		(SEQ ID NO:178) (SEQ ID NO:179)
6	F GGCCTGTGTACACCACCAACCA C R CTCGTAACGTGACGTGTTGGT	214	67		(SEQ ID NO:180) (SEQ ID NO:181)
6	F CATGATCTGCGGTGTGCTCTA R GCAGCCGAGCCACAGCAAT	267	67	61.5, 62.0	(SEQ ID NO:182) (SEQ ID NO:183)

In high-throughput analysis, samples from three patients were pooled. PCR was performed with a thermal cycler (iCycler, Bio-Rad; Hercules, CA) in a total volume of 20  $\mu$ l containing; 45 ng of genomic DNA, 2  $\mu$ l GeneAmp 10x PCR buffer II, 2  $\mu$ l of GeneAmp dNTP mix with a 2.0 mM concentration of each dNTP, 2.4  $\mu$ l of a 25 mM MgCl<sub>2</sub> solution; 4 pmol of each primer, and 0.1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The PCR conditions were; denaturation at 95° C for 9 min, followed by 35 cycles at 95° C for 1 min, 65° C or 67° C for 30 sec (Table 1), and 72° C for 1 min and 30 sec, and a final extension step at 72° C for 7 min.

### Denaturing HPLC Analysis

For high-throughput analysis, a 25  $\mu$ l volume of PCR products from the three patients was automatically injected into the chromatograph for analysis using the WAVE<sup>®</sup> System for DHPLC analysis (Transgenomic, Omaha, NE). The DHPLC melting temperatures are listed in Table 41.

When abnormal chromatographic patterns were detected in the pooled samples by the high-throughput protocol, the sample was reanalyzed individually in the WAVE<sup>®</sup> System. The PCR product that showed the abnormal chromatographic pattern was then sequenced.

### Direct DNA Sequencing

For direct sequencing, PCR products were purified with a QIA Quick PCR purification kit (Qiagen, Valencia, CA) to remove unused primers and precursors. The PCR products were directly sequenced with the same forward and reverse PCR amplification primers on an ABI310 automated sequencer using BigDye chemistry according to the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA).

### Screening Myocilin Gene

Two patients with glaucoma who harbored the mutation in the Noelin 2 gene were screened in the myocilin gene by DHPLC.

**Genotyping Noelin 2 c.462G>A (Arg144Gln) Polymorphism**

The G to A substitution at position c.462 in exon 4 of the Noelin 2 gene was detected by using restriction enzyme, BstU1. The G allele sequence was cut into two  
5 fragments (140 bp + 200 bp) by BstU1, while the A allele sequence remained intact (344 bp).

The polymorphism was confirmed by restriction-enzyme assay and by the chromatographic pattern of DHPLC.

**Statistical Analyses**

10 The frequencies of the genotypes and alleles in patients and controls were compared with the chi-square test or Fisher's exact test. The Hardy-Weinberg equilibrium for the observed frequencies was also calculated. Statistical analysis was performed with SPSS program (SPSS  
15 Inc., Chicago, USA ). A P value of <0.05 was considered to be significant.

**Results****Noelin 2 Variants in Japanese Subjects**

20 A total of 616 Japanese subjects were studied, and the results are presented in Table 42. Ten sequence changes were identified in the glaucoma patients and control subjects. Among these, two were missense changes, seven were synonymous codon changes, and one was a change in intron sequences. One possible disease causing-mutation,



Arg144Gln, was identified in one POAG proband and one POAG proband, and was not present in the 300 normal Japanese controls. No significant difference was detected between glaucoma patients and controls for the Arg106Gln ( $P=0.30$ ),  
 5 Ala226Ala ( $P=0.30$ ), and Arg427Arg ( $P=0.30$ ).

The NTG patient with Arg144Gln harbored the Arg76Lys change in the myocilin gene.

A possible glaucoma-causing mutation in exon 4, Arg144Gln, was identified in 2(0.3%) of the 616 Japanese  
 10 glaucoma patients.

Table 42. OLFM2 Variants observed in glaucoma patients and control subjects

Location	Sequence Changes	Codon Changes	Frequency in Subjects (%)		
			POAG	NTG	Control
Exon 4	c.462G>A	Arg144Gln	1 / 276 (0.4)	1 / 340 (0.3)	0 / 300 (0)
Exon 3	c.348G>A	Arg106Gln	111 / 211 (52.6)	135 / 276 (48.9)	115 / 241 (47.7)
Exon 3	c.289G>A	Thr86Thr	1 / 211 (0.5)	0 / 276 (0)	0 / 241 (0)
Exon 3	c.346G>A	Ala105Ala	1 / 211 (0.5)	0 / 276 (0)	0 / 241 (0)
Exon 4	c.451G>A	Lys140Lys	1 / 276 (0.4)	0 / 340 (0)	0 / 300 (0)
Exon 4	c.487G>A	Glu152Glu	2 / 276 (0.7)	0 / 340 (0)	0 / 300 (0)
Exon 5	c.628C>T	Thr199Thr	0 / 211 (0)	1 / 274 (0.4)	0 / 241 (0)
Exon 5	c.709G>A	Ala226Ala	15 / 211 (7.1)	27 / 274 (9.9)	28 / 241 (11.6)
Exon 6	c.1312C>T	Arg427Arg	34 / 211 (16.1)	45 / 270 (16.7)	30 / 240 (12.5)
Intron 6	c.1393+42T>C		117/210 (55.7)	N/C	N/C

\* Sequence variation was found by direct sequencing analysis.

15

Partial nucleotide sequence of Noelin 2 comprising the targeted polymorphisms is as follows:

Noelin 2 codon 144(underlined) CCG(Arg) to CAG(Gln) : (GG:

200 bp+144 bp, GA: 344 bp+200 bp+144 bp, AA: 344 bp)

(BstUI)

codon 140(underlined) Lys140Lys (AAG>AAA)

5 codon 152(underlined) Glu152Glu (GAG>CAA)

79741 ttagttccta caatggagtc atgtctggga agaattctagg gtccaatatg agccacatgt  
79801 caagggccag gtgtgcatca aagacaaagg gtgaagtat gagtcagagg ttggagtcatt  
79861 gtctgggtca aaggccagggt gtcaggcttg gccatggctc catcttgatg cacaggagct  
10 79921 gaaggacagg atgacggaac tgttgccctt gagctcggct ctggagcagt acaaggcaga  
79981 caccgggacc attgtacgct tcggggagga ggtgaggaat ctctccggca gctggcgccg  
80041 cartcaggag gagatgggtg cctacgggta tgaggacctg cagcaacggg tgatggccct  
80101 ggaggcccggt ctccacgctt gcgccagaa gctgggtatg ccttgccctt tgaccctgac  
80161 ccctgatctc tgactgccac acccaactcc agtatcacct gtttgtgcct agaagctgga  
15 80221 cacagttttg acctetaact tttaaacttc aacccttgac ctctctacct aaggctacac  
(SEQ ID NO:184)

79841-79862, 80164-80184; primers for detecting  
polymorphism at codon 144

79916-80131, coding region

20 Example 13. Evaluation of the Heat shock protein 70-  
1(HSP70-1) gene in the etiology of glaucoma

Association between glaucoma and gene polymorphism  
of HSP70-1 (Biogerontology 4: 215-220, 2003 and Hum Genet  
114: 236-241, 2004) was examined among POAG, NTG patients

and control subject using Invader assay.

The primary probes (wild and mutant probes) and Invader<sup>®</sup> oligonucleotides (Invader<sup>®</sup> probe) used to detect the polymorphism of HSP70-1 gene are shown in Table 43.

5

Table 43. The oligonucleotide sequence of HSP70-1

Gene	Polymorphism	nucleotide change	format	Probe	Sequence	
HSP70-1	-110A>C	A to C	PCR	A	Flap sequence-TTTTCGCCTCCCGT	(SEQ ID NO:185)
				C	Flap sequence-GTTTCGCCTCCCGT	(SEQ ID NO:186)
				Invader	GCTGCCAGGTCGGGAATAITCCAGGGC	(SEQ ID NO:187)
			PCR	F	CGCCATGGAGACCAACACC	(SEQ ID NO:188)
				R	GCCGGTTCCCTGCTCTCTGTC	(SEQ ID NO:189)

#### Results

As shown in Table 44, the polymorphism of -110A>C in  
10 HSP70-1 is associated with glaucoma, especially FOAG.

Table 44. Genotype distribution and allele frequency of  
HSP70-1 gene polymorphisms in glaucoma patients and  
controls

HSP70-1-110A/C														
	Genotype Frequency			P	AC/CC			P	AA/AC			Allele frequency		P
	AA	AC	CC		AA	AC	CC		AA	G	G			
CONTROL	67	130	44		67	174			197	44		284	218	
	241	27.8	13.3		27.8	72.2			18.3	81.7		54.8	43.2	
NTG	108	130	54	0.069	108	184	82.4	0.032	298	54	18.5	342	238	0.169
	230	32.6	44.9		32.6	67.4			16.6	83.4		80.0	41.0	
POAG	84	94	33	0.026	84	127	83.4	0.007	178	33	0.460	282	180	0.026
	211	39.8	44.5		39.8	89.2			84.4	15.6		85.1	37.9	
GLAUCOMA	130	224	87	0.020	190	311	0.007	0.007	414	87	0.765	604	398	0.044
	501	37.9	44.7		37.9	82.1			82.8	17.4		80.3	38.7	

Partial nucleotide sequence of HSP70-1 comprising the  
targeted sequence is as follows:

HSP70-1 -110A>C (the following sequence is the C allele.)

61 cctccagtga atcccagaag actctggaga gttctgagca gggggcgcca ctctggcctc  
121 tgattgggtcc aaggaaggct ggggggcagg acgggaggcg aaagccctgg aatattcccg  
181 acctggcagc etcatcgagc tcggtgattg gctcagaagg gaaaaggcgg gtctccgtga  
241 cgacttataa aacgccaggg gcaagcggtc cggataacgg ctagcctgag gagctgctgc  
5 301 gacagtccac tacctttttc gagagtgact cccgttgctc caaggcttcc cagagcggaac  
(SEQ ID NO:190)

**Example 14. Evaluation of the Endothelin converting enzyme  
1(ECE1) gene in the etiology of glaucoma**

10 Association between glaucoma and gene polymorphism  
of ECE1 was examined in POAG and NTG patients using Invader  
assay.

The primary probes (wild and mutant probes) and  
Invader<sup>®</sup> oligonucleotides (Invader<sup>®</sup> probe) used to detect  
15 the polymorphism of ECE1 gene are shown in Table 45.

Table 45. The oligonucleotide sequence of ECE1

Gene	Polymer/heteronucleotide charge	Target	format	arm	Probe	Sequence	Length (bp)	Tm (°C)	Dye
ECE1	C-33A	C to A	PCR	1-3	C	Flap sequence- GTGGCCGAGAGCA	23	63.0	FAM
					A	Flap sequence- TTGGCCGAGAGCA	26	63.2	RED
					Invader	GCGAGATACAAAGATAGGGAAGGTGGCGTCAATG	37	77.5	
					PCR	F TAAGTCGGGCTTCAACAGG			
					R	AAAGTGAAGATAGGCAATTAATG			

(SEQ ID NO:132)  
(SEQ ID NO:133)  
(SEQ ID NO:134)  
(SEQ ID NO:135)

## Results

As shown in Table 46, the polymorphism of -338C>A in ECE1 is associated with high IOP in NTG.

Table 46. Genotype distribution of ECE-1 gene polymorphisms in glaucoma patients and controls

ECE-1/-338C>A polymorphism		three genotypes					
Clinical characteristics		CC	n	CA	n	AA	n
POAG	Age at diagnosis (yr)	56.8 ± 12.2	68	57.8 ± 12.4	108	61.8 ± 10.5	34
	IOP at diagnosis (mm Hg)	26.2 ± 5.8	60	26.8 ± 6.5	94	26.6 ± 4.8	32
	Visual field score at diagnosis	3.1 ± 1.0	68	3.1 ± 0.9	108	3.0 ± 0.8	35
NTG	Age at diagnosis (yr)	58.1 ± 13.0	97	54.2 ± 12.2	138	54.1 ± 14.2	53
	IOP at diagnosis (mm Hg)	18.7 ± 2.4	91	16.8 ± 2.4	123	15.6 ± 2.6	46
	Visual field score at diagnosis	2.8 ± 0.7	99	2.8 ± 0.7	138	2.8 ± 0.7	53

  

ECE-1/-338C>A polymorphism		two genotypes					
Clinical characteristics		CC	n	CA/AA	n	CC+CA	n
POAG	Age at diagnosis (yr)	56.8 ± 12.2	68	59.8 ± 12.1	146	57.4 ± 12.3	174
	IOP at diagnosis (mm Hg)	26.2 ± 5.8	60	26.7 ± 6.1	128	26.5 ± 6.2	154
	Visual field score at diagnosis	3.1 ± 1.0	68	3.0 ± 0.9	140	3.1 ± 0.9	173
NTG	Age at diagnosis (yr)	59.1 ± 13.0	97	54.1 ± 12.8	183	56.2 ± 12.7	233
	IOP at diagnosis (mm Hg)	18.7 ± 2.4	91	16.5 ± 2.5	189	16.7 ± 2.4	214
	Visual field score at diagnosis	2.8 ± 0.7	99	2.8 ± 0.7	189	2.8 ± 0.7	235

Partial nucleotide sequence of ECE-1 comprising the targeted polymorphism is shown as follows:

ECE1 -338C>A (underlined)

1 ttttgtctgg tctttctagc attaaccccc tagacacacc taaggctgat gccgggggga  
5 61 acctgtcttg attgctctgg gccagatcga gggcaccttc ctgatacttt tgttatctgc  
121 cactggggac cgggttggtg aagggggact taagattttc tcgaaggagg ggtcactgtg  
181 agggcctttc ctgcctgcta ggggcttcag ttggggggcc cccactcccg actccgggca  
241 agggaggggt ccccatctcc cccgggcctc tcgggtcttg gggctctccc gggaggccgg  
(SEQ ID NO:196)

10

Example 15. Evaluation of the CD50 gene in the etiology of open-angle glaucoma

Polymorphism of CD50 gene was identified using polymerase chain reaction-restriction fragment length  
15 polymorphism (PCR-RFLP) techniques (Table 47).

20



Table 47. Primer sequences, product size, and annealing temperatures

Gene	Primer sequences (5' to 3')	primer name	Product size (bp)	Annealing temperature (°C)	Restriction Enzyme	(SEQ ID NO:197)
CD95	C T A C G T A G A G C T A T C T A C G G T T C	CD95F	232	65.0	Mva I	(SEQ ID NO:198)
(A-4700)	G G C T G T C C A T G T T G T G G C T G C	CD95R				(SEQ ID NO:199)

As shown in Table 48, the polymorphism of A-670G in CD95 is associated with glaucoma, especially POAG.

[illegible][illegible]

**Example 16. Evaluation of the EPHX1 gene in the etiology of glaucoma**

Association between glaucoma and gene polymorphism of EPHX1 was examined among POAG, NTG patients and control  
5 subject using Invader assay.

The primary probes (wild and mutant probes) and Invader<sup>®</sup> oligonucleotides (Invader<sup>®</sup> probe) used to detect the polymorphism of ECE1 gene are shown in Table 49.

10

15

20

Table 49. The oligonucleotide sequence of

Mutation	nucleotide change	Target	Probe	Sequence	Length	Tm	Dye	USDA ID NO. 2.9	USDA ID NO. 3.0	USDA ID NO. 3.1
EPH1 K119	G to A	Sense	Wild	Flap sequence-CTTATCTTGAGTCAGGG	25	62.7	FM	USDA ID NO. 2.9	USDA ID NO. 3.0	USDA ID NO. 3.1
			Mutant	Flap sequence-TTATCTTGAGTCAGGG	31	62.3	RED	USDA ID NO. 2.9	USDA ID NO. 3.0	USDA ID NO. 3.1
			Header	TGTGTGGTGGGATTTGGAGAGAGGTTGAA	30			USDA ID NO. 2.9	USDA ID NO. 3.0	USDA ID NO. 3.1

**Results**

As shown in Table 50, the polymorphism of G>A in codon 119 Lys is associated with glaucoma, especially NTG.

Table 50. Genotype distribution and allele frequency of  
EPHX1 gene polymorphisms in glaucoma patients and  
controls

	Genotype frequency		P	G/G		P	G/h+A/A		P	G/G+G/A		P	A/A		P	Allele frequency		P
	G/G	G/A		G/G	G/A		G/h+A/A	G/h+A/A		G/G+G/A	G/G+G/A		A/A	A/A		G	A	
CONTROL	107	87		107	30		117	184		184	30		301	147		301	147	
NTG	121	110	0.100	121	19		123	231		231	19	0.039	352	148		352	148	0.286
POAG	83	84	0.669	83	29		93	147		147	29	0.388	230	122		230	122	0.583
	178	171		178	165		178	331		331	165		653	347		653	347	

Partial nucleotide sequence of EPHX1 comprising the  
targeted polymorphisms is as follows:

ccagGACTTA CACCAGAGGA TCGATAAGTT CCGTTTCACC  
 CCACCTTTGG AGGACAGCTG CTTCCACTAT GGCTTCAACT  
 CCAACTACCT GAAGAAAGTC ATCTCTACT GCGGGAATGA  
 ATTTGACTGG AAGAAGCAGG codon 113 (T/C)  
 codon 119 (G/A) TGGAGATTCT CAACAGATAC  
 CCTCACTTCA AGACTAAGAT TGAAGgtatg ttgtc aaaac  
 gccagccaga gagggatgta tgtcatgaga acagccttct  
 primer 1  
 primer 2  
 primer 3 (SEQ ID

Example 17. Evaluation of the  $\beta 2$  adrenergic receptor (ADRB2) gene in the etiology of glaucoma

5 Association between glaucoma and gene polymorphism  
of ADRB2 was examined in open angle glaucoma patients (POAG  
and NTG patients) using Invader assay.

The primary probes (wild and mutant probes) and Invader® oligonucleotides (Invader® probe) used to detect the polymorphism of ADRB2 gene are shown in Table 51.

Table 51. The oligonucleotide sequence of ADRB2

Gene	Mutation	nucleotide change	Target	Probe	Sequence	Length (bp)	T <sub>m</sub> (°C)	Dye
ADRB2	Gln194G (G46A)	G to A	Sense	A	Flap sequence-TATTGGGTGGCGCA	24	63.8	RED
				C	Flap sequence-CATTGGGTCCGCG	24	63.2	FAM
				Inverted	TGGTGGTCCGCGCTTGGCTTCA	23	71.5	
				C	Flap sequence-GAAGGGGACGAGGTGT	28	63.8	RED
ADRB2	Gln270Gln (G79Q)	C to G	Anti-Sense	G	Flap sequence-GAAGGGGACGAGGTGT	30	63.4	FAM
				Inverted	GGTGGGACGAGGAGTCCGCGAGT	23	77.0	

(SEQ. ID. NO. 233)  
(SEQ. ID. NO. 234)  
(SEQ. ID. NO. 235)  
(SEQ. ID. NO. 236)  
(SEQ. ID. NO. 237)  
(SEQ. ID. NO. 238)



**Results**

As shown in Table 52, the polymorphism of Gly16Arg (G46A) of ADRB2 is associated with early onset of POAG.

5 Table 52. Clinical characteristics of glaucoma patients according to genotype of Gln16Arg in the ADRB2 gene

ADRB2	Gly16Arg	Genotype		P value*
		RR	RG+GG	
OAG	Age at diagnosis (ys)	57.9 ± 12.7 (n=100)	56.3 ± 12.7 (n=371)	0.085
	IOP at diagnosis (mm Hg)	20.3 ± 5.8 (n=90)	20.8 ± 6.5 (n=335)	0.469
	Visual field score at diagnosis	2.8 ± 0.7 (n=99)	2.9 ± 0.8 (n=375)	0.508
POAG	Age at diagnosis (ys)	62.9 ± 12.7 (n=39)	56.7 ± 11.7 (n=162)	0.001
	IOP at diagnosis (mm Hg)	26.3 ± 4.9 (n=33)	26.3 ± 6.0 (n=147)	0.973
	Visual field score at diagnosis	3.0 ± 0.9 (n=38)	3.1 ± 0.9 (n=164)	0.898
NTG	Age at diagnosis (ys)	54.7 ± 11.7 (n=61)	56.0 ± 13.5 (n=209)	0.531
	IOP at diagnosis (mm Hg)	16.8 ± 2.5 (n=57)	16.6 ± 2.4 (n=188)	0.581
	Visual field score at diagnosis	2.7 ± 0.5 (n=61)	2.8 ± 0.7 (n=211)	0.266

P value\* with Logistic regression analyses

10 As shown in Table 53, the polymorphism of Gln27Glu (C79G) is associated with high intraocular pressure (IOP) in OAG, especially POAG.

Table 53. Clinical characteristics of glaucoma patients according to genotype of Gln27Glu in the ADRB2 gene

ADRB2	Gln27Glu (Q27E)	Genotype		P value*
		QQ	QE+EE	
POAG	Age at diagnosis (ys)	58.4 ± 12.3 (n=162)	56.3 ± 12.2 (n=30)	0.272
	IOP at diagnosis (mm Hg)	26.0 ± 5.1 (n=144)	28.6 ± 9.1 (n=28)	0.039
	Visual field score at diagnosis	3.1 ± 0.9 (n=163)	3.1 ± 0.9 (n=30)	0.837
NTG	Age at diagnosis (ys)	55.6 ± 12.8 (n=250)	58.2 ± 12.6 (n=23)	0.986
	IOP at diagnosis (mm Hg)	16.6 ± 2.5 (n=230)	17.1 ± 2.0 (n=17)	0.447
	Visual field score at diagnosis	2.8 ± 0.7 (n=251)	2.8 ± 0.6 (n=24)	0.692
OAG	Age at diagnosis (ys)	56.7 ± 12.7 (n=412)	57.1 ± 12.3 (n=53)	0.448
	IOP at diagnosis (mm Hg)	20.2 ± 5.9 (n=374)	24.2 ± 9.2 (n=45)	<0.001
	Visual field score at diagnosis	2.9 ± 0.8 (n=414)	2.9 ± 0.8 (n=54)	1.000

\*P value with Logistic regression analyses

Partial nucleotide sequence for ADRB2 gene  
containing the targeted polymorphisms is as follows:

ADRB2 codon Nos. Gly16Arg(GGA>AGA): Gln27Glu ( CAA>GAA )  
(underlined)

```
5      1 gcgcttacct gccagactgc gcgccatggg gcaacccggg aacggcagcg ccttcttgct
      61 ggcacccaat ggaagccatg cgccggacca cgacgtcacg cagcaaaggg acgaggtgtg
     121 ggtggtgggc atgggcatcg tcatgtctct catcgtcctg gccatcggtg ttggcaatgt
     181 gctggtcacg acagccattg ccaagttcga gcgtctgcag acggtcacca actacttcat
     241 cacttcactg gctgtgtctg atctggtcac gggcctagca gtggtgccct ttggggccgc
    10    301 ccatattctt atgaaaaatgt ggacttttgg caacttcttg tgcgagtttt ggacttccat
      (SEQ ID NO:209)
```